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EDITORIAL COMMENT

INSECT PHYSIOLOGY, BIOCHEMISTRY AND PHARMACOLOGY: AN IN DEPTH REVIEW

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In July 1980 a meeting was held at the Pergamon Press office in Oxford to discuss the publication of a comprehensive series of volumes on insect physiology, biochemistry and pharmacology. The idea was to produce 12 volumes that would provide an up to date summary and orientation on insect physiology, biochemistry, pharmacology and control, that would be essential to research workers and teachers.

Larry Gilbert and myself were asked to act as Executive Editors and by December 1980 we had produced a 27 page booklet giving the aims and objectives for the project, together with details of the proposed 12 volumes. This booklet was sent to the authors for each chapter and their comments invited. By the middle of 1981 most of the chapters were assigned to authors and the work was under way. The preliminary booklet gave details of suggested layout of chapters, abbreviations, preparations of diagrams and tables, journal citations etc. to ensure that there would be uniformity of presentation.

The 12 volumes are as follows :

Volume 1. Embryology and Reproduction
Volume 2. Postembryonic Development
Volume 3. Integument, Respiration and Circulation
Volume 4. Regulation: Digestion, Nutrition, Excretion, Thermoregulation
Volume 5. Nervous System: Structure and Motor Function
Volume 6. Nervous System: Sense Organs and Integration
Volume 7. Endocrinology I
Volume 8. Endocrinology II
Volume 9. Behaviour
Volume 10. Biochemistry
Volume 11. Pharmacology
Volume 12. Insect Control: Pesticides, Biological Control, Resistance.

Larry Gilbert was the Co-ordinating Editor for Volumes 1, 2, 3, 7, 8 and 10. I was the Co-ordinating Editor for Volumes 4, 5, 6, 9, 11 and 12.

In addition, there is a final volume, Volume 13, which is the Index volume. Although each volume will contain its own subject index, species index and author index, Volume 13 will contain the subject index, species index and author index for all 12 volumes so that any material in these 12 volumes will be rapidly located.

An illustrated brochure on these volumes is available from Pergamon Press.

All references in the volumes are given with full titles of papers, journal volume and first and last pages of the references. The references to the authors in the text are given with their full initials so that it is clear that the text refers to D. S. Smith and not, say, to A. D. Smith. There are more than 50,000 references to literature, more than 35,000 different species of insect referred to and all will be readily found in the 12 different volumes.

There are 240 authors of the 200 chapters in the volumes and they have produced a series of very readable, up to date, critical summaries of the literature. In addition they have been able to give insight into the problems associated with their subject, indicate the present state of the subject and where it will be going over the next decade.

With so many authors involved in the project there were bound to be epidemiological problems. Many of the older ones had heart attacks, many of the younger ones had matrimonial problems, and the ones who were middle-aged were peripatetic and always going to, or coming from, Australia/South America/China, so that letters and manuscripts were chasing them around the world in a clockwise direction. Nevertheless, the chapters came in to the Editors, revisions were made, galley proofs were sent out and returned and the volumes are now being printed.

This series of volumes will be indispensible to major libraries but an important case can be made that they should also be considered as research instruments. A set should be available in the laboratory for reference. They will then provide the research workers with an up to date account of the literature and will always be instantly available. For this reason they should be considered equally as important as a research microscope, oscilloscope or spectrophotometer. The volumes will save research workers at least a month each year, in that not only will they provide an awareness of the literature and so save valuable research time, but the full index to authors, subject and species will also make it much easier to write reports and papers on their own new research work.

In many cases libraries have special grants for the purchase of such sets of volumes. Also, it is often possible to purchase these volumes against a research equipment grant.

It is hoped that these volumes will do much to strengthen the case for insects as the source of research material, not only because insects are important medical and agricultural pests (over 200 million people at present have malaria; insects eat or destroy about 20% of planted food crops), but also because insects in many cases are the ideal unique research material for studying fundamental biological problems.

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MINIREVIEW

RESPIRATORY PHYSIOLOGY OF BURROWING MAMMALS AND BIRDS

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(*Received* 13 *April* 1983)

The advantages of living and/or nesting in burrows that offer protection from predation and climatic extremes have resulted in the widespread use of this niche by a great many animals. In modeling gas exchange in animal burrows, both Wilson and Kilgore (1978) and Withers (1978) found that diffusion through the soil is the primary mechanism of gas exchange, but that this may not be sufficient in the burrows of most endotherms to avoid large gradients of respiratory gases (i.e. substantial elevations of burrow CO_2 concentrations above and reductions of burrow O_2 concentrations below that of outside air). Field measurements of gas concentrations within the occupied burrows of birds and mammals bear this out. (For review of mammals see Tenney and Boggs (1983); for gas concentrations of avian burrows see Birchard *et al.* (1983), Wickler and Marsh (1981).) The CO_2 may be as much as 6% or more while the O_2 is generally about 14%.

Faced with the chronic hypoxia and hypercarbia of the burrow atmosphere, it would seem incumbent upon burrow-dwelling birds and mammals to make certain physiological adjustments to avoid excessive energy expenditure in respiratory work while still insuring adequate O_2 delivery to their tissues and coping with the potential CO_2-perturbation of their acid–base balance. The adaptations such animals have made are only beginning to be understood. Burrowing mammals have been studied considerably more than burrowing birds but in this review we will summarize the literature on both classes looking for common patterns as well as diversity of response and directions for further investigation.

VENTILATION

Since hypoxia and hypercapnia are both strong respiratory stimulants, it is of interest to examine the ventilatory responses of burrow-dwelling mammals and birds who may be chronically exposed to these two stimuli. In several fossorial and semi-fossorial mammals the ventilatory response to CO_2 has been well studied and is clearly reduced compared to non-fossorial species (Fig. 1). The greatest reduction in CO_2-sensitivity is observed in the completely fossorial pocket gopher (*Thomomys bottae*) (Darden, 1972) and mole rat (*Spalax ehrenbergi*) (Arieli and Ar, 1979), but the semi-fossorial echidna (*Tachyglossus aculeatus*) (Parer and Hodson, 1974), chipmunk (*Tamias striatus*) (Maclean, 1978) and woodchuck (*Marmota monax*) (Boggs and Birchard, 1984) also show significantly reduced ventilatory responses to CO_2 compared to the human (Darden, 1972) or porcupine (*Erethizon dorsatum*) (Boggs and Birchard, 1984) (Fig. 1).

The difference in ventilatory set-point suggested by the CO_2-response curves is also suggested by the low resting or air-breathing ventilation, low arterial P_{O_2} and high arterial P_{CO_2} observed in some burrowing mammals. The pocket gopher and mole rat both have lower than predicted ventilations while breathing room air. The pocket gopher's breathing frequency is 80% of the predicted value, the mole rat's frequency is 60% of predicted and its ventilation is 70% of predicted (Darden, 1972; Arieli and Ar, 1979). The low ventilation in the pocket gopher is associated with a lower than predicted oxygen consumption and therefore it has only a slightly elevated Pa_{CO_2} of 44–45 Torr, but the metabolism of the mole rat is normal and its calculated Pa_{CO_2} is 48 Torr. Table 1 presents blood gas data from the strictly fossorial pocket gopher and three species of semi-fossorial mammals, while breathing room air. The arterial P_{O_2} values of 60–75 Torr are significantly lower than the common mammalian value of 90–95 Torr, and the Pa_{CO_2} values of 45–53 are higher than the usual value of approximately 40 Torr. The elevated Pa_{CO_2} is usually accompanied by an elevated bicarbonate concentration [HCO_3^-] such that an arterial pH of roughly 7.4 is preserved. Such is the case for the ground squirrel (*Citellus lateralis*), echidna and pocket gopher (Table 1) (Chapman and Bennet, 1975). The woodchuck, however, presents an exception to that pattern with an apparent lack of renal compensation of its respiratory acidosis.

The burrowing owl (*Athene cunicularia*) and the bank swallow (*Riparia riparia*) are the only burrow-dwelling avian species that have been studied to date. The burrowing owl has been shown to exhibit a significantly higher threshold to its ventilatory response to CO_2 than any of several nonburrowing avian species (Fig. 2) (Boggs and Kilgore, 1983). Bank swallows also show a reduced ventilatory amplitude and frequency response to inspired CO_2 compared to the closely related non-burrowing cliff swallow

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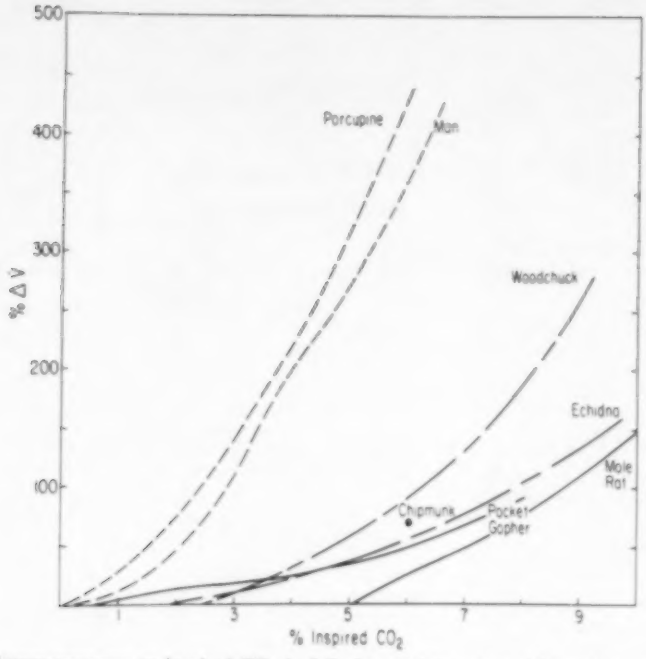

Fig. 1. Ventilatory responses to inspired CO_2 in fully fossorial (——), semi-fossorial (— — —) and non-fossorial (- - - -) mammals. Data from Arieli and Ar (1979) on mole rats, Darden (1972) on pocket gophers and man, Maclean (1978) on chipmunks, Parer and Hodson (1974) on echidnas, Boggs and Birchard (1983) on woodchucks and porcupines.

(Furilla, 1980). Blood gas data are not presently available for either of these small burrowing birds, but neither the air-breathing ventilation nor oxygen consumption were below predicted in the burrowing owl (Boggs and Kilgore, 1983).

The ventilatory response of burrow-dwelling mammals and birds to hypoxia has been investigated less than the CO_2 response. Arieli and Ar (1979) found that the threshold of the hypoxic ventilatory response of the completely fossorial mole rat was at an inspired O_2 of about 8% which is lower than that of the white rat, man, cat or llama (Laciga and Koller, 1976; Lahiri, 1974; Brooks and Tenney, 1968). The semi-fossorial woodchuck and echidna, on the other hand, have a hypoxic ventilatory threshold at an inspired O_2 of approximately 11–12% which is the same as that observed in the similar sized non-burrowing porcupine (Boggs and Birchard, 1984; Parer and Hodson, 1974). Hence, a reduced ventilatory sensitivity to hypoxia is not as common a characteristic among burrowing mammals as is a reduced sensitivity to CO_2. The hypoxic ventilatory response has only been studied in one burrow-dwelling bird. The threshold of the response to hypoxia was significantly lower in the burrowing owl than in the similar sized bobwhite or several other non-burrowing birds (Boggs and Kilgore, 1983).

OXYGEN TRANSPORT

Adjustments in the oxygen transport properties of the blood of burrow-dwelling mammals and birds do not conform to a consistent pattern. Some mammalian burrowers (e.g. moles and pocket gophers) show an increase in oxygen capacity or content of their blood (Bartels *et al.*, 1969; Quilliam, *et al.*, 1971; Chapman and Bennet, 1975; Lechner, 1976; Jelkmann *et al.*, 1981) either through an elevation of hemoglobin levels, increases in erythrocyte numbers, or changes in blood volume. Elevated blood hemoglobin concentrations have been reported in moles, pocket gophers, and in a semi-fossorial murid rodent (Withers, 1975), while hemoglobin concentrations in other burrowing mammals, e.g. naked mole rats, mole rats and woodchucks, are not different from similar sized non-burrowing rodents (Table 2). Hemoglobin concentrations in the

Table 1. Average blood gas values while breathing air

Species	PaO_2 (mmHg)	$PaCO_2$ (mmHg)	pHa	HCO_3^- (mEq/L)	Reference
Thomomys bottae (pocket gopher)		45	7.381	28.1	Darden (1972); Chapman and Bennet (1975)
Citellus lateralis (ground squirrel)	75.3	52.4	7.418	33	Burlington *et al.* (1969)
Tachyglossus aculeatus (echidna)	60.5	53	7.429	33	Parer and Hodson (1974)
Marmota monax (woodchuck)	72	48	7.357	26	Boggs and Birchard (1984)

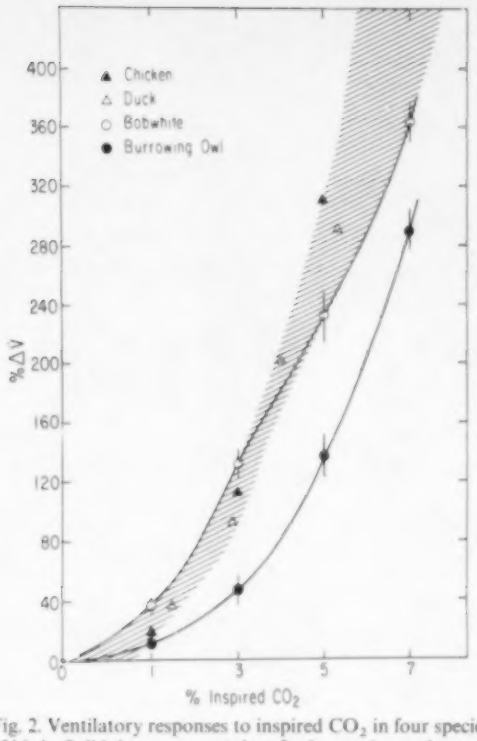

Fig. 2. Ventilatory responses to inspired CO_2 in four species of birds. Solid dots represent data for burrowing owls, open dots for bobwhite (Boggs and Kilgore, 1983), solid triangles for brown leghorn chickens (Johnston and Jukes, 1966) and open triangles for Muscovy (Powell *et al.*, 1978) and Aylesbury (Jones and Purves, 1970) ducks.

few burrow-dwelling birds studied to date are not significantly elevated in comparison to other birds (Table 2). Although the bank swallow hematocrit and hemoglobin appear to be high they are not significantly different from those of the closely related barn swallow (Kilgore and Birchard, 1980).

Ar *et al.* (1977) observed a marked polycythemia in the mole rat, which was due to a lowered mean cell volume (36 μm^3) rather than an increased hematocrit. They suggested smaller cells may be advantageous since the oxygenation rate constant is inversely related to size of erythrocytes (Ar *et al.*, 1977). On the other hand an elevated hematocrit in the mole is associated not with an increased number of red blood cells but a rather large mean cell volume (100 μm^3) (Quilliam *et al.*, 1971). Contact time is probably more important than erythrocyte size in oxygenation, but it is interesting to find such differences in red cell volume between two completely fossorial species.

The extent to which oxygen transport to the tissues of burrowers is influenced by alterations in blood volume and cardiac output is largely unknown. Quilliam *et al.* (1971) cite an article reporting a blood volume in moles of approximately twice that of other rodents, on a per unit body mass basis. Cardiac output has been estimated in the mole rat to be about 0.34 of that of similar sized white rats breathing air (Ar *et al.*, 1977). Parer and Metcalfe (1967) likewise found the cardiac output of the burrowing echidna to be about 0.25 that of a rabbit of similar body mass. The low heart rates of some burrowers at normoxia, coupled with an elevated heart rate during exposure to hypoxia (Arieli and Ar, 1981a) and a larger estimated arterial-venous O_2 difference, suggests that some burrowing mammals may have greater cardiac reserves than nonburrowers.

The most common adjustment in the oxygen transport path in burrowing mammals is an elevated hemoglobin O_2-affinity (lower P_{50}) (Table 2). This higher affinity persists at normal body temperatures in those species with lower-than-normal temperatures (mole rats, naked mole rats and echidnas) (Johansen *et al.*, 1976; Ar *et al.*, 1977; Parer and Metcalfe, 1967). A high oxygen affinity of hemoglobin can be achieved via one or a combination of three possible mechanisms: (1) a high intrinsic oxygen affinity of hemoglobin; (2) a low level of 2,3-diphosphoglycerate (2,3-DPG) in red cells; and (3) a reduced interaction of hemoglobin with 2,3-DPG. The first of these mechanisms accounts for the increased affinity of the blood of the naked mole rat (Johansen *et al.*, 1976), the second that of the armadillo (Dhindsa *et al.*, 1971) and the latter for that of moles (Jelkmann *et al.*, 1981).

A higher than predicted hemoglobin O_2-affinity is not a consistent characteristic of the blood of burrow-dwelling birds. The bank swallow P_{50} was significantly lower than the barn swallow P_{50} or the allometrically predicted value, but the P_{50} of the burrowing owl was not significantly less than that of a similar sized bird and was actually higher than the allometric prediction (Boggs *et al.*, 1983; Kilgore and Birchard, 1980).

Most mammalian burrowers seem to maintain the CO_2-Bohr effect of their blood within the normal range for small mammals with the exception of the woodchuck whose CO_2-Bohr factor of -0.72 is quite high (Table 2). The burrow-dwelling birds do not show any deviation from the normal range of Bohr factors in birds.

Other adjustments in the oxygen path in burrowing mammals may include a reduced tissue diffusion distance (Arieli and Ar, 1981b), elevated levels of muscle myoglobin (Lechner, 1976; Ar *et al.*, 1977), an exaggerated A–V O_2 difference (Ar *et al.*, 1977) and reductions in tissue metabolism (e.g. see McNab, 1966). The capillary density of skeletal and cardiac muscle in mole rats is twice that of the white rat (Arieli and Ar, 1981b), yielding shorter calculated diffusion distances that are 70% that of the white rat. Myoglobin levels in skeletal muscles of mole rats and pocket gophers are, respectively, 3 and 2 times those in white rats (Lechner, 1976; Ar *et al.*, 1977). Interestingly, myoglobin levels in the cardiac muscle of both species were not significantly different from those of the white rat.

McNab (1966) and others (e.g. Bentley *et al.*, 1967; Dhindsa *et al.*, 1971; Kay, 1979) have found that a number of species of fossorial rodents exhibit lower body temperatures and metabolic rates than expected on a body mass basis. Yet others find no deviation from the expected metabolic rate (Ar *et al.*, 1977; Kay, 1977) or even a higher oxygen consumption (and lower thermal conductance) than expected (Gettinger, 1975). McNab (1979) has recently re-examined the question of low rates of metabolism in fossorial mammals and now notes that basal metabolic rates are lower than expected from mass in fossorial mammals with masses

Table 2. Blood characteristics of some burrowing mammals and birds

	Weight (g)	Hct (vol %)	Hb (g/100 ml)	P_{50} (Torr)	P_{50} Predicted*	$\Delta \log P_{CO_2}/\Delta$ pH	$\Delta \log P_{50}/\Delta$ pH
Mammals							
Chipmunk (*Tamias striatus*) (Maclean, 1978, 1981)	~70	48.9				−2.04	
Pocket gopher (*Thomomys bottae*) (Chapman and Bennet, 1975; Lechner, 1976)	135	46	17.1	33.3	39	−2.67	−0.61
Mole (*Talpa europaea*) (Quilliam *et al.*, 1971; Bartels *et al.*, 1969)	~140	48.7	17.4	16	~38.5		−0.47
Naked mole rat (*Heterocephalus glaber*) (Johansen *et al.*, 1976)	105–106	46	13.6	23.3	35–39		−0.43
Mole rat (*Spalax ehrenbergi*) (Ar *et al.*, 1977)	196	45.6	15.0	29.5	37.9	−1.32	−0.53
Ground squirrel (*Spermophilus beecheyi*) (Baudinette, 1974)	598	48.7	15.1	26	35.6		
Prairie dog (*Cynomys ludovicianus*) (Hall, 1965)	1280	47.3	15.1	22	34.1		
Echidna (*Tachyglossus aculeatus*) (Parer and Metcalfe, 1967)	1680	48	17.6	21.3	33.7		−0.49
Woodchuck (*Marmota monax*) (Boggs and Birchard, 1984)	4450	39.4	13.5	27.8	32	−1.29	−0.72
Birds							
Bank swallow (*Riparia riparia*) (Kilgore and Birchard, 1980)	11.7	50.7	17.0	37.7	45.6	−0.968	−0.546
Burrowing owl (*Athene cunicularia*) (Boggs *et al.*, 1983)	144	36.6	12	44.9	37.4	−1.39	−0.412

*Predicted values from Schmidt-Nielsen and Larimer (1958) for mammals, Lutz *et al.* (1974) for birds.

in excess of 80 g, but are higher than expected in those with masses less than 60 g. This conclusion apparently also holds for semi-fossorial mammals.

There have been no studies of tissue diffusion distances, myoglobin levels, or A–V oxygen differences in burrow-dwelling birds. However, data on the metabolic rate of at least one burrow dweller, the burrowing owl, suggests that they have metabolic rates in air equivalent to those predicted from their body size (Boggs and Kilgore, 1983).

BLOOD BUFFERING

There is some evidence to suggest that at least a few fossorial mammals, but no burrow-dwelling birds, have increased their reliance on blood buffering of CO_2. Both the semi-fossorial chipmunk and the completely fossorial pocket gopher have a greater average non-carbonic buffering strength than that of the white rat (−2.67 and −2.04 $\Delta \log P_{CO_2}/\Delta$ pH respectively vs −1.39 $\Delta \log P_{CO_2}/\Delta$ pH in the rat (Table 2)). The pocket gopher also has a greater bicarbonate concentration. The elevated non-carbonic buffering strength of the pocket gopher is due to significantly greater amounts of both hemoglobin and organic phosphates than observed in non-fossorial rodents (Lechner, 1976). The non-carbonic buffering strength of mole rats and woodchucks, on the other hand, is not different from that of white rats (Table 2). Ar *et al.* (1977) have interpreted this lack of an improved buffering capacity of the blood of mole rats coupled with normal CO_2-Bohr factor to indicate that under hypoxic–hypercapnic conditions the mole rat has forsaken the maintenance of a constant blood pH to obtain more oxygen by using the Bohr shift.

Blood buffering (non-carbonic buffering strength) in the burrowing birds studied to date is not different from non-burrowing birds, nor for that matter from mammals. Non-carbonic buffering strength of burrowing owl blood was $-1.39\,\Delta \log P_{CO_2}/\Delta$ pH and that of bank swallows was −0.97 (Table 2).

INTERPRETATIONS AND COMPARISONS

A reduced ventilatory responsiveness to CO_2 is ubiquitous among burrowing mammals and birds and could represent an energy conserving strategy since an increased ventilation may be of little utility in eliminating CO_2 when the inspired CO_2 is high. There may be differences between birds and mammals, however, in the mechanisms behind that reduced ventilatory responsiveness to CO_2. The reduced response to CO_2 of the burrowing owl was due to an altered threshold whereas in some of the burrowing mammals a difference in the slope of the response was detectable as well as a difference in threshold. The mammals often exhibited a reduced air-breathing ventilation as well, with an associated elevation of Pa_{CO_2} and depression

of Pa_{O_2}, and in most, but not all, cases an elevated $[HCO_3^-]$. We do not know what the burrowing birds' blood gases and bicarbonate concentrations are, but since their ventilation is not depressed it seems unlikely that they share the elevated Pa_{CO_2} observed in burrowing mammals.

The altered ventilatory responsiveness to CO_2 in burrowing mammals could reflect differences in chemoreceptor characteristics as well as in blood buffering properties. Human CO_2-retainers, such as patients with emphysema, exhibit the same pattern of elevated Pa_{CO_2} and $[HCO_3^-]$ and reduced sensitivity to inspired CO_2 (Tenney, 1954). Normal humans acclimatized to CO_2 also show an elevated $[HCO_3^-]$ and a reduced ventilatory sensitivity to inspired CO_2 (Schaeffer *et al.*, 1963). In most burrowers, as in human "CO_2-retainers", some fraction of the reduced ventilatory responsiveness to CO_2 may be attributable to the elevated buffer base (Tenney, 1954). But other adaptations to CO_2 at the cellular level of the central chemoreceptors may also have taken place, particularly in burrowing mammals, who show such greatly reduced responses to CO_2 and some of whom, e.g. the woodchuck and mole rat, are operating at low blood and tissue pH (Boggs and Birchard, 1984; Ar *et al.*, 1977).

Although there are many similarities in the respiratory control systems of birds and mammals an important difference *vis-à-vis* CO_2 is the presence of intrapulmonary chemoreceptors in birds that appear to respond to intrapulmonary P_{CO_2} directly, i.e. not via changes in extracellular $[H^+]$ (see Bouverot, 1978, for review). It thus seems likely that the altered threshold of the owl's CO_2 response reflects some adjustments at the level of the lungs or in the central integration of afferent information from the lungs. A difference in dead space and breathing pattern was noted in the owl (Boggs and Kilgore, 1983; Boggs *et al.*, 1983) and may contribute to a lower intrapulmonary P_{CO_2} and higher intrapulmonary P_{O_2} at the same level of inspired CO_2 or O_2 compared to the other species. However, an alteration in the discharge characteristics of the intrapulmonary CO_2-receptors is another possibility.

A reduction in the ventilatory response to hypoxia compared to other species of similar size would seem, of necessity, to be associated with varying degrees of adjustment in other components of the O_2 delivery system such as the shape and position of the oxyhemoglobin dissociation curve, tissue capillary density and myoglobin concentrations, and the magnitude of the cardiac output response to hypoxia. The relatively reduced hypoxic ventilatory responsiveness of the mole rat has proven to be associated with all the aforementioned adjustments in the O_2 delivery system (see above). More information on these other aspects of the O_2-delivery system is needed on other mammalian burrowers. The burrowing owl also showed a reduced ventilatory response to hypoxia but no increase in hemoglobin O_2-affinity. It therefore would seem particularly important for this bird to employ an increased blood flow response to hypoxia. The relatively large heart size of the burrowing owl (Boggs *et al.*, 1983; Hartman, 1955) is at least suggestive of the potential for substantial elevations of cardiac output. This needs to be studied directly. A large cardiac output has been pointed to as one aspect of the better hypoxic tolerance of birds compared to mammals (Tucker, 1968) and of the bar-headed goose, a high altitude species, compared to the lowland Pekin duck (Black and Tenney, 1980).

REMAINING QUESTIONS

Aside from the questions already raised about alterations in chemoreceptor function and quantitative differences in cardiac output responses to hypoxia in burrowers, some additional questions arise from the presently available information on burrowing birds and mammals.

In their study of European bee-eaters (*Merops apiaster*) White *et al.* (1978) measured high levels of ammonia gas in the litter-filled nests as well as relatively high CO_2 and low O_2 concentrations. The ammonia levels were over 100 ppm on average and could exceed 700 ppm in the extreme. Few mammals can tolerate ammonia gas, which can be very irritating to eyes and airways and can have either excitatory or inhibitory effects on respiration. Scant information on poultry suggests birds also suffer ill-effects from ammonia gas over 50–100 ppm (Charles and Payne, 1966; Caveny and Quarles, 1978). We need to learn more about the tolerance of birds for ammonia gas, particularly among burrowing birds who do not clean their nests. Some cave-dwelling bats show a remarkable tolerance for ammonia and part of their tolerance is due to CO_2 retention which tends to counter the alkaline excess caused by NH_3 hydration (Studier and Fresquez, 1969). Perhaps the elevated CO_2 in the nests of burrowing birds helps to some extent to counter the deleterious effects of the NH_3.

Another rather fundamental question arising from the studies of burrowing birds and mammals is, how are the ventilatory "set points" set? Is a reduced CO_2 sensitivity a genetically determined and selected trait, or is it a phenotypic characteristic that can be ontogenetically determined by the environment through an acclimitization process that is only very slowly reversed? In the pocket gopher (Darden, 1972), mole rat (Arieli and Ar, 1979) and the burrowing owl (Boggs and Kilgore, 1983) studies, the animals were held in captivity, outside of the burrow atmosphere, for 60 days or more prior to study and the subsequent data on ventilatory responsiveness to CO_2 was taken, therefore, not to reflect acclimitization phenomena. This assumption of "deacclimatization", however, is based largely on studies of CO_2 acclimatization and deacclimatization rates in adult humans. Yet it seems quite possible that chronic exposure to elevated environmental CO_2 pre-natally as well as post-natally could have a more profound acclimitization effect that would have a very much slower decay than acclimitizations to relatively short-term exposures in the adult. The reduced ventilatory responsiveness to hypoxia observed in high-altitude residents does not appear to be a genetic but rather an acquired characteristic that is lost very slowly upon subsequent residence at sea-level (Lahiri *et al.*, 1976; Dempsey and Forster, 1982). Similarly, the ventilatory adaptation to CO_2 observed in burrowers could be an acquired rather than an inherent characteristic. However, unlike the blunted hypoxic response of high altitude residents that is not apparent at birth, a reduced CO_2 sensitivity perhaps

could be established pre-natally as a result of the exposure to elevated P_{CO_2} throughout development. There are more mechanisms to insure adequate oxygenation of fetal tissues when the mother lives in a hypoxic environment than there are mechanisms to insulate the fetus from elevated P_{CO_2} as a result of the mother's exposure and adjustment to a high CO_2 atmosphere. The question of whether or not pre-natal exposure to elevated P_{CO_2} results in a reduced CO_2 sensitivity in the adult is presently being pursued.

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EFFECTS OF CORTISOL AND ACTH ON GILL Na^+/K^+-ATPase, SDH AND CHLORIDE CELLS IN JUVENILE ATLANTIC SALMON *SALMO SALAR* L.

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Abstract—1. ACTH, but not cortisol, stimulated the gill Na^+/K^+-ATPase activity of *S. salar*.

2. Both ACTH and cortisol increased the SDH activity in a suspension of gill cells, from pre-smolts, but not in a homogenate of gill tissue.

3. Neither hormone increased the size or abundance of gill chloride cells.

4. ACTH caused fin darkening but neither ACTH nor cortisol induced body silvering.

INTRODUCTION

The corticosteroid hormones from the interrenal glands have been implicated in the regulation of parr–smolt transformation in the Atlantic salmon *Salmo salar* L. because during this phase there is a rise in serum cortisol levels (Langhorne and Simpson, 1981) and histological evidence of activation of the pituitary–interrenal axis (Olivereau, 1975). Two well-recognised features of the transformation are the rise in gill Na^+/K^+-ATPase activity (Saunders and Henderson, 1978) and silvering of the body by deposition of purines in the dermis (Johnston and Eales, 1967). The observations that cortisol treatment stimulated the gill Na^+/K^+-ATPase activity and induced body silvering in yellow (freshwater) eels (*Anguilla spp.*) (Epstein *et al.*, 1971; Forrest *et al.*, 1973) suggest that cortisol could be responsible for similar changes in the parr–smolt transformation of salmon.

We therefore measured gill Na^+/K^+-ATPase activity in juvenile Atlantic salmon treated with cortisol or adrenocorticotropin (ACTH). We also measured the size and abundance of the gill chloride cells, and the activity of the mitochondrial-marker enzyme, succinic dehydrogenase (SDH). Cortisol treatment is reported to cause proliferation of the chloride cells and their mitochondria in eel gills (Doyle and Epstein, 1972), and both Na^+/K^+-ATPase and SDH activities have been shown to be preferentially located in the chloride cells in the eel gill (Sargent *et al.*, 1975).

MATERIALS AND METHODS

Animals and hormone treatments

Freshwater Atlantic salmon juveniles were transferred from the Smolt Rearing Station, Almondbank, Scotland, to Stirling, and maintained on a commercial salmon diet for at least 2 weeks before experimentation. Experiments were carried out on 10-month-old (mo) parr, 12-month-old pre-smolts, and fully-silvered 25-month-old smolts. The terms parr, pre-smolt and smolt are used in a restricted sense to describe fish showing no silvering, partial and complete silvering, respectively. Age is given in months, arbitrarily from the first day of April. Hormone-treated fish received cortisol (0.8 or 4.0 mg/kg) or ACTH (100 or 250 IU/kg) in 0.1 ml of sterile 0.9% (w/v) NaCl, and sham-injected controls received 0.1 ml of 0.9% NaCl, by daily intraperitoneal injection between 2 and 4 p.m. Brief sedation in 20 ppm quinaldine was used to facilitate gentle handling.

Chemicals

Cortisol as the sodium salt of hydrocortisone-21-phosphate, adrenocorticotropin (Grade II, porcine), Na_2ATP (Grade I), ouabain, Fiske and Subbarow reducer, bovine serum albumen, and *para*-iodonitrotetrazolium violet (INT) were obtained from Sigma (London) Chemical Co. Ltd., Poole, Dorset, UK. Analytical grades of all other reagents were obtained from BDH Chemicals Ltd., Poole, Dorset, UK. All solutions were prepared in glass-distilled water.

Analytical methods

Enzyme and protein assays were carried out in duplicate. Fish were killed by pithing about 20 hr after the last treatment, and the gills perfused with chilled 0.9% (w/v) NaCl via the ventral aorta. The gill epithelium was removed from the gill arches by scraping with a blunt scalpel blade on cold glass, and transferred into about 10 vol of cold (2–4°C) homogenizing medium composed of 10 mM Na_2 EDTA, 300 mM sucrose and 10 mM 2-mercaptoethanol, in 100 mM imidazole–HCl buffer at pH 7.2. The tissue was homogenized with 12 complete strokes at 350–400 rpm in a power-driven Teflon-in-glass homogenizer held in ice. The $Na^+/K^+/Mg^{2+}$-ATPase activity in 60–100 μg of homogenate protein was determined in a 1 ml reagent mixture containing 100 mM NaCl, 20 mM KCl, 5 mM $MgCl_2$, and 5 mM Na_2ATP, in 100 mM imidazole–HCl buffer at 35°C and pH 7.2, for 20–30 min, after a 5 min incubation at 35°C, in a shaking water-bath. Residual or Mg^{2+}-ATPase activity was determined in a similar system from which KCl was omitted, in the presence of 0.5 mM ouabain. The reaction was terminated by adding 0.4 ml 10% (w/v) sodium dodecyl sulphate and the determination of inorganic phosphate (P_i) liberated from ATP completed by the method of Peterson (1978). The specific activity of Na^+/K^+-ATPase was calculated as the difference in rate of phosphate release between the two reagent mixtures, per mg protein. Protein levels were determined by the method of Lowry *et al.* (1951) using bovine serum albumen as standard.

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SDH activity was also determined in the fresh homogenate, according to Clark and Porteous (1964) as the rate of reduction of 0.07% (w/v) INT to formazan (F) in the presence of 50 mM succinate, at 20°C and pH 7.2, per mg protein.

A suspension of gill cells in a modified Hanks saline was prepared by passing gill scrapings through a syringe needle to mechanically dissociate the gill cells, and also assayed for SDH by the INT method, as detailed by Sargent *et al.* (1975). Differential cell counts were carried out on a sample of cell suspension reacted with INT and succinate in a haemocytometer under a Nomarski interference microscope. Chloride cells were identified as large (12–26 μ), roughly spherical (in cell suspensions), cells with a "ground-glass" appearance to their cytoplasm which displayed an intense granular red staining reaction for SDH activity. Quadruplicate counts on 100–150 cells were averaged for each fish, and the estimate considered reliable when the four counts fell within 10% of their mean. The abundance of chloride cells was expressed as a percentage of total cells present, excluding erythrocytes. The mean diameter of 20 chloride cells was estimated by means of an ocular graticule.

RESULTS

Salmon smolts (25 months old) receiving 0.8 mg/kg or 4.0 mg/kg cortisol for 14 days showed no significant changes in specific activity of Na^+/K^+-ATPase (Table 1). The high dose decreased the yield of gill protein, which inflated the calculated values for specific activity. The total Na^+/K^+-ATPase activity was significantly decreased by treatment at both dose rates (Table 1).

Table 2 gives the results of cortisol (0.8 mg/kg) and ACTH (100 or 250 IU/kg) injection in 10-month-old parr and 12-month-old pre-smolts for 8 and 14 days respectively. Specific activities only are shown since at these dose rates the calculation of total activities led to the same conclusions. ACTH produced a 60–70% increase in Na^+/K^+-ATPase activity at either dose rate, but cortisol had no significant effect.

SDH activity in the homogenates was unaltered by either hormone treatment. Both ACTH and cortisol however increased the SDH activity in the cell suspensions after 14 days in pre-smolts, but not after 8 days in parr. Cortisol produced a 100% increase, and ACTH a 65% increase, compared with the sham-injected control values. The sham-injected controls had less whole-cell SDH activity than the unhandled controls, but the levels produced by cortisol and ACTH exceeded those of both control groups (Table 2).

The Na^+/K^+-ATPase activity in the homogenates and the SDH activity in the cell suspensions were unaltered 6 hr after a single high dose of cortisol (200 mg/kg) in pre-smolts (Table 3).

Neither cortisol nor ACTH altered the % yield or size of the chloride cells in the cell suspensions (Table 4).

DISCUSSION

Prolonged cortisol treatment did not stimulate the gill Na^+/K^+-ATPase activity in freshwater salmon (Tables 1 and 2). A similar lack of response has been noted in freshwater specimens of the euryhaline mullet, *Chelon labrosus* (Risso) (Gallis *et al.*, 1979). In eels (Epstein *et al.*, 1971; Forrest *et al.*, 1973) and hypophysectomized *Fundulus heteroclitus* (Linn.) (Pickford *et al.*, 1970), however, daily injections of cortisol at 0.5–4.0 mg/kg stimulated several-fold increases in gill Na^+/K^+-ATPase activity after 7–10 days. The response to cortisol treatment thus varies between species. This may indicate differences in the physio-

Table 1. Effect of high and low doses of cortisol on gill Na^+/K^+-ATPase activity and gill protein content after 14 days treatment in 25-month smolts

Treatment	n	Na^+/K^+-ATPase		Gill protein content
		Specific activity (μmol P_i/mg prot/hr)	Total activity (μmol P_i/g fish/hr)	(% of controls)
Sham-injected controls	7	4.55 ± 0.31	3.37 ± 0.27	100 ± 6
Cortisol (0.8 mg/kg)	7	3.72 ± 0.23	2.65 ± 0.19*	96 ± 6
Cortisol (4.0 mg/kg)	6	3.95 ± 0.38	2.52 ± 0.25*	81 ± 5*

Data are means ± SEM.
* Significantly different from controls at $P < 0.05$ (t-test).

Table 2. Effects of ACTH and cortisol on specific activities of gill Na^+/K^+-ATPase, and SDH in gill homogenates and cell suspensions, from salmon parr (8 days treatment) and pre-smolts (14 days treatment)

Treatment	Na^+/K^+-ATPase in homogenate (μmol P_i/mg prot/hr)	SDH activity in homogenate (nmol Formazan/mg prot/hr)	SDH activity in cell suspension (nmol Formazan/mg prot/hr)
Parr (Feb, 10 month)			
Unhandled controls	1.23 ± 0.22	90.0 ± 5.5	71.0 ± 2.7
Sham-injected controls	1.15 ± 0.18	85.5 ± 4.3	48.1 ± 1.4††††
Cortisol (0.8 mg/kg)	1.36 ± 0.28	84.0 ± 1.8	59.7 ± 5.9
ACTH (100 IU/kg)	1.95 ± 0.27†	95.3 ± 9.4	57.6 ± 5.4
Pre-smolts (Apr, 12 month)			
Unhandled controls	2.27 ± 0.20	83.8 ± 7.4	94.2 ± 4.4
Sham-injected controls	2.07 ± 0.41	74.2 ± 6.8	68.0 ± 3.8††
Cortisol (0.8 mg/kg)	2.73 ± 0.37	71.1 ± 4.9	133.9 ± 5.4†††*
ACTH (250 IU/kg)	3.96 ± 0.21††	73.9 ± 3.3	112.6 ± 5.8†**

Data are means ± SEM, $n = 6$. † Significantly different from sham-injected controls* or unhandled controls† at † $P < 0.05$, †† $P < 0.01$, ††† $P < 0.001$ (t-test).

Table 3. Na^+/K^+-ATPase activity in homogenates and SDH activity in cell suspensions 6 hr after a single high dose of cortisol in 11 month pre-smolts (means ± SEM)

Treatment	n	Na^+/K^+-ATPase (μmol P_i/mg prot/hr)	n	SDH (nmol F/mg prot/hr)
Controls (saline)	4	2.03 ± 0.24	3	69.1 ± 10.8
Cortisol (200 mg/kg)	4	2.20 ± 0.25	3	73.7 ± 11.4

Data are means ± SEM, no significant differences between means (t-test).

logical role or pharmacological effect of cortisol in these species, or in the timing of the test treatments (cf. Meier and Fivizzani, 1980).

The response also varied with the developmental stage of the fish, as observed in *C. labrosus* (Gallis *et al.*, 1979). Prolonged or short-term treatment with cortisol had no effect on the Na^+/K^+-ATPase activity in salmon parr or pre-smolts (Tables 2 and 3) but caused a significant depression of total activity in fully-silvered smolts (Table 1). The specific activity (expressed per mg protein) was not decreased in the latter case, partly because of the concurrent decrease in gill protein content (Table 1).

ACTH however clearly stimulated the gill Na^+/K^+-ATPase activity (Table 2), as reported in the eel (Milne *et al.*, 1971). Since porcine ACTH (as used here) has been shown to cause cortisol release in salmon (Fagerlund, 1970) the discrepancy between the effects of ACTH and cortisol is difficult to explain. Direct stimulation of the enzyme by ACTH, or stimulation by ACTH-released hormones other than cortisol, cannot be discounted. It also remains possible that the effect of ACTH was mediated by cortisol, and that the direct administration of cortisol itself was ineffective for pharmacological reasons.

The stimulation of gill Na^+/K^+-ATPase activity by ACTH was not dose-related and was relatively small (60–70%) when compared to the several-fold increases in activity induced by ACTH (Milne *et al.*, 1971) and cortisol (Epstein *et al.*, 1971) in eels. Furthermore the response to cortisol in eels was accompanied by the synthesis of new, mitochondria-rich chloride cells (Doyle and Epstein, 1972). Neither ACTH nor cortisol affected the number or size of the chloride cells in cell suspensions from salmon gills (Table 4). These hormones thus appear to make only a limited contribution to regulating the gill Na^+/K^+-ATPase activity in the chloride cells of salmon, in marked contrast to their strongly anabolic effects in the eel.

Both ACTH and cortisol stimulated the SDH activity in the whole-cell suspensions, without increasing the SDH activity in the homogenates, when administered to pre-smolts (Table 2). The mechanism was presumably an increase in the functional rate, in the intact cell, of the mitochondrial pathways in which SDH participates, rather than increased synthesis of SDH which would have also increased the content of SDH activity in the homogenates. The pituitary–interrenal axis may regulate gill SDH activity by this mechanism since the same effect occurred, and to a similar extent, during the subsequent parr-smolt transformation of this population (unpublished results). Stimulation of the whole-cell SDH activity was apparent after 14 days treatment in pre-smolts, but not after 8 days in parr (Table 2), or 6 hr after a single high dose of cortisol (Table 3). Either the effect was slow to develop or fish in the parr stage were less responsive than pre-smolt fish entering the parr-smolt transformation phase.

Table 4. Effect of 14 day treatments with ACTH or cortisol on size and % yield of chloride cells in a suspension of gill cells from 12 month pre-smolts

Treatment	Mean diameter (μ, n = 3 fish)	% Yield (n = 6 fish)
Sham-injected controls	18.6 ± 0.7	9.7 ± 0.8
Cortisol (0.8 mg/kg)	19.7 ± 0.3	8.1 ± 0.4
ACTH (250 IU/kg)	19.5 ± 0.5	8.3 ± 0.7

Data are means ± SEM, no significant differences between means (t-test).

The depression of whole-cell SDH activity by the handling and injection procedure was presumably not a cortisol-mediated stress response, since cortisol or ACTH treatment reversed the effect, and induced higher levels than were seen in either unhandled or sham-injected fish (Table 2).

The silvering of the body produced by cortisol treatment in eels (Epstein *et al.*, 1971) was not observed in salmon receiving cortisol or ACTH treatment in the present work. In the salmon pre-smolts ACTH, but not cortisol, caused darkening of the dorsal surface and fins, apparent 24 hr after the first injection, and increasing until the dorsal fin, pectoral fin and trailing edge of the caudal fin were densely speckled with black pigment after 7 days of treatment. Only slight darkening occurred at the lower dose rate in parr. Similar changes are a well-known feature of parr-smolt transformation in the Atlantic salmon and occur naturally in our hatchery populations during April and May.

It was also noted that the fish receiving cortisol or ACTH became less excitable and were more easily netted than the sham-injected controls after 5–7 days. A similar effect of cortisol treatment has been noted in the eel and likened to the state of reduced activity seen in migratory-phase silver eels (Epstein *et al.*, 1971). The high serum cortisol levels noted in Atlantic salmon smolts (Langhorne and Simpson, 1981) may then contribute to the reduced swimming ability seen in Atlantic salmon smolts (Thorpe and Morgan, 1978) compared with parr (Wankowski and Thorpe, 1979) and also reported in coho salmon *Oncorhynchus kisutch* Walbaum smolts (Smith, 1982).

The results in this paper suggest the pituitary–interrenal axis is only partly responsible for the rise in Na^+/K^+-ATPase activity seen in smolting salmon, the regulation of which may be more complex than in some other teleost species, notably the eels. The stimulation of whole-cell SDH activity and the behavioural changes caused by ACTH and cortisol, and the fin-darkening induced by ACTH, indicate other

possible functions of the activated pituitary–interrenal axis (Olivereau, 1975; Langhorne and Simpson, 1981) in smolting Atlantic salmon.

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MAJOR INORGANIC COMPONENTS IN SOME FRESHWATER CRUSTACEANS (MALACOSTRACA), THEIR EXUVIAE AND FOOD ITEMS, AND THERMOGRAVIMETRIC–DIFFERENTIAL THERMAL ANALYSIS OF ORGANIC AND ASH COMPONENTS

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Abstract—1. Thermogravimetric and differential thermal analysis was used to examine changes in mass of whole-bodies and exuviae of *Gammarus pulex*, *G. lacustris*, *Asellus aquaticus* and *Austropotamobius pallipes*, at temperatures between 60 and 1000°C.

2. 500°C was the best temperature for ashing these organisms, ensuring combustion of organic components whilst minimizing decomposition of carbonates, but sodium and chloride were volatilized from some materials at 500°C. The mass of the organic fraction was therefore overestimated and inorganic ash was underestimated, by *ca.* 5–10% for *Gammarus* materials.

3. A mass balance for inorganic anions equalled 76–86% of cations in exuviae and 67–77% in whole-bodies of *Gammarus* and *Asellus*. Cold extraction and hot digestion methods gave different estimates of the total amounts of chloride and sodium.

INTRODUCTION

The cuticle of crustaceans is similar to that of insects but in many it also contains large amounts of calcium salts deposited as a calcified layer in the endocuticle (Dennell, 1947, 1960; Digby, 1967, 1968; Welinder, 1974, 1975a,b). These calcium salts are partly mobilized at the moult, with very large losses of calcium, up to 96% of body calcium in a freshwater amphipod, *Gammarus pulex*, and 90% in a freshwater crayfish, *Austropotamobius pallipes*, of which more than half is shed in the cast skin. The losses are replaced by rapid uptake of calcium from the external medium immediately after the moult (Drach, 1939; Dall, 1965; Chaisemartin, 1967; Graf, 1964, 1968; Vincent, 1969; Greenaway, 1974a,b,c; Wright, 1979, 1980a,b). Most of the calcium is precipitated as carbonate, and recent studies on large marine decapods state that calcium carbonate in the cuticle is almost exclusively present as crystalline calcite (Richards, 1951; Dennell, 1960; Travis, 1963) although earlier work indicates that in some cases the carbonate is amorphous; calcium is also present as amorphous calcium phosphate (Vinogradov, 1953). Both amorphous and crystalline forms of carbonate probably occur in freshwater crustaceans (Vinogradov, 1953; Reid, 1943; Chaisemartin, 1967).

Although there is extensive literature on the chemical composition of the cuticle of crustaceans, most of it is fragmented and the emphasis is on organic components; there are relatively few complete chemical analyses for inorganic components in freshwater crustaceans. None of these show the balance between cations and anions and there are scant data of this kind even for marine crustaceans (Clarke and Wheeler, 1922; Richards, 1956). We have attempted to provide such data for two common freshwater amphipods, *Gammarus pulex* (L.) and *G. lacustris* Sars, comparing analyses on moulted skins (exuviae) with whole-body contents. A knowledge of the inorganic components in exuviae is essential for estimating total ion budgets during growth and for studying the effects on ion uptake when the chemical composition of the external environment is altered. We also give results for an isopod, *Asellus aquaticus* (L.), and a crayfish (Decapoda), *Austropotamobius pallipes* Lereboullet.

The investigations reported here were prompted by three requirements and a curious observation. Requirements were: (1) measurement of organic (ash free) and ashed dry weights of exuviae and body contents of *Gammarus*, (2) determination of the major inorganic components in the ash, (3) confirmation that most if not all of the calcium (and magnesium) in exuviae is present as carbonate rather than in some other form, e.g. hydroxyapatite, associated with relatively large amounts of phosphate (Needham, 1954; Richards, 1956). Dry materials were therefore ashed at 500°C (for 5 hr), the standard temperature recommended to minimize or prevent decomposition of carbonate (Grodzinski *et al.*, 1975). Surprisingly, when ashed at this temperature, *Gammarus* exuviae retained the shape and form previously found after drying in air at 60°C. We had expected the ash to crumble into powder and therefore needed to ascertain that all of the carbonized organic matrix had been combusted. Grove *et al.* (1961) report that some carbon remained when cattle serum was ashed at 500 and 550°C. Lafon (1943, p. 133) refers to the presence of residual carbon in crustacean material even after prolonged heating at a mean temperature of 500°C. In order to select an ashing temperature that would give complete (or near-complete) combustion of organic components whilst minimizing losses of inorganic components, we employed combined thermogravimetric (TG) and dif-

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ferential thermal analysis (DTA) (Wendlandt, 1975). TG graphically records changes in weight (mass) of a sample when heated in a small furnace in a pre-programmed manner under controlled conditions. Simultaneously, DTA records any difference in temperature between the sample and a reference compound in the furnace. The direction of heat flow is given, indicating whether reactions are endothermic or exothermic at any particular temperature or range of temperatures, and it is then possible to calculate the quantity of heat that has been emitted or absorbed by the sample. The results are reported herein.

For comparison with TG–DTA on crustacean exuviae we also analysed whole-bodies of intermoult animals and items of food used in the laboratory: a filamentous alga (*Nitella*) and leached elm leaves.

MATERIALS AND METHODS

Animals were collected locally and held for brief periods in the laboratory before analysis of whole-body contents. Others were kept at 15°C for collection of exuviae. The latter, and whole animals, were air-dried in an oven at 60°C until constant weight was attained after 1–3 days. Food items of *Gammarus* were treated in the same manner, using *Nitella flexilis* (L.) Agardh (Willoughby, 1983) and fallen leaves of smooth-leaved elm (*Ulmus carpinifolia* Gleditsch) that had been leached in running tap water for various lengths of time, termed 2-day and 14-day leached elm (Sutcliffe *et al.*, 1981). Whole crayfish (B) and their exuviae (E), minus the large chelipeds, provided sufficient dry mass for analyses to be done on individuals. The heavily chitinized and mineralized chelipeds were removed and treated separately, as whole chelipeds (C) and cheliped exuviae (EC). For *Gammarus* and *Asellus*, whole bodies (B) and exuviae (E) of 30–50 animals were pooled in each sample. Three to five samples were used for chemical analysis. Other pooled samples were used for examination by TG–DTA.

Samples of dried materials (60 ± 5°C) were ground to a fine powder with an agate pestle and mortar. Sub-samples were taken for analysis of individual elements. On some materials, replicate analyses for sodium and chloride were done (Table 4) after extraction of dry solids in cold deionized water, extraction in cold deionized water after ashing in a muffle furnace at 500 ± 50°C or fusion with sodium carbonate to red heat at 970°C. Sulphur was determined as sulphate, with the turbidimetric method of Tabatabai (1974), on the solution obtained after acid extraction of the residual ash from TG–DTA after heating to 1000°C. Total carbonate and residual carbonate were respectively measured on dry solids (60°C) and ash (1000°C), using a modification of the method given by Stainton (1973); sulphuric acid was added to the sample in a closed vial and the volume of liberated CO_2 was measured by gas liquid chromatography. Total silicon was determined with molybdenum blue, and aluminium by atomic absorption spectrophotometry, after sodium carbonate fusion of dry solids (Mackereth *et al.*, 1978). Chloride was measured by silver nitrate titration with an Aminco-Cotlove Titrator (Sutcliffe *et al.*, 1982). The major cations and phosphorus were measured on dried samples which had been digested in concentrated nitric acid followed by perchloric acid at 90°C; volumes were made up to 100 ml with deionized water. Phosphorus was determined with molybdenum blue (Mackereth *et al.*, 1978). Major cations were analysed on a Perkin Elmer 2380 Atomic Absorption Spectrophotometer. Background correction was used for magnesium and iron; 1% lanthanum chloride was added for magnesium and calcium.

A Stanton Redcroft STA 780 Simultaneous TG-DTA system (Durn, 1980) was used for thermogravimetric (TG) and differential thermal analysis (DTA). Samples of dried material (60°C) were ground to powder and passed through a 100 μm sieve. Al_2O_3 was used as the reference material for measuring temperature changes due to exothermic and endothermic reactions in samples of *ca.* 20 mg dry wt, in an atmosphere of flowing air. In isothermal studies, the temperature was raised from ambient to the experimental levels at rates of 20°C min^{-1}; preprogrammed temperatures of 450, 500 and 550°C were held constant (within ± 1°C) for 15–20 hours. In other studies the temperature was raised continuously from ambient to 1000°C at 20°C min^{-1}.

RESULTS

TG–DTA at rising temperatures

All of the materials examined had four main regions of energy change on the DTA trace (Figs 1 and 2), representing either exothermic or endothermic reactions. These occurred at approximately the same ranges of temperatures for the different materials, as indicated below. Table 1 shows the relative amounts of solids remaining at the end of each region of energy change, expressed as percentages of the initial dry weight of solids in material previously dried at 60°C.

(i) *Endothermic region* (*ca.* 60–250°C). Weight losses (TG) of *ca.* 6–12% were presumably due to progressive

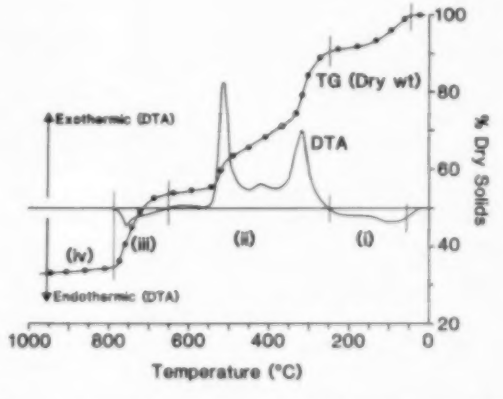

Fig. 1. Drawing (semi-diagrammatic) from a recorded TG–DTA trace of crayfish exuviae showing relative peak heights for DTA and percentage change in dry mass (TG). (i)–(iv) indicate major regions of energy change.

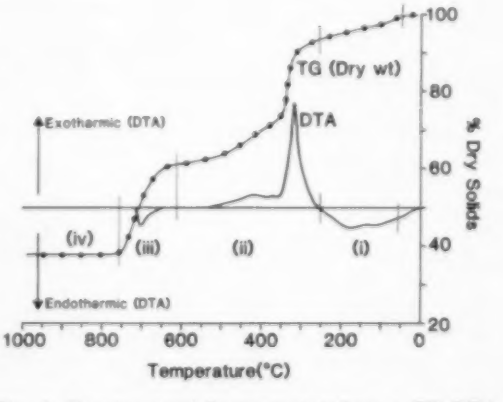

Fig. 2. Drawing (semi-diagrammatic) from a TG–DTA trace of *G. lacustris* exuviae. Details as in Fig. 1.

Table 1. Percentage dry solids remaining at the end of DTA regions (i) to (iv). Means of 1–4 observations

Material	End of (i)	End of (ii)	End of (iii)	End of (iv)
Exuviae				
Crayfish (E)	88	51	30	28.5
Crayfish (EC)	89	55	34.5	33
G. pulex (E)	91	64	37	37
G. lacustris (E)	93.5	62	38	37
Asellus (E)	89.5	65	39.5	39
Whole body				
Crayfish (B)	89	32	14	12
Crayfish (C)	91.5	41	22	19.5
G. pulex (B)	93.5	22	15	14
G. lacustris (B)	91.5	21.5	14.5	14
Asellus (B)	92	27.5	18	17.5
Plants				
Nitella	91	12.5	12.5	8
2-day leached elm	91	9	6.5	6.5
14-day leached elm	91.5	9	6.5	6

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evaporation of chemically-bound water and volatile organic components.

(*ii*) *Exothermic region* (*ca.* 250–650°C). Relatively large losses in dry weight represent the combusion of organic substances. These naturally comprise a much greater proportion of dry solids in whole-body and plant materials compared to the smaller proportions in animal exuviae. Region (ii) displayed the biggest differences between various types of material. Crayfish (B, C, E, EC), represented by results shown in Fig. 1, differed from all other materials, represented by results shown in Fig. 2, in having four peaks (one very small) on DTA at temperatures of *ca.* 320, 420, 520 and 620°C, with relative peak heights of 0.60, 0.18, 1.00 and 0.03 respectively. Other materials had three peaks, at *ca.* 320, 370 and 420°C, with relative peak heights of 1.0, 0.02 and 0.02 respectively in *G. lacustris* exuviae (Fig. 2). Differences between the number and heights (=relative energy change) of DTA peaks in animal materials may represent (a) chemical fractionation, where a variety of organic components burn off at different times, and/or (b) physical interference in the burning process, caused by varying rates of oxygen transport into and removal of combustion products from a rigid structure of chitin and calcite. Simple calculations based on the calorific values of chitin and protein (Richards, 1951, 1953), and the percentage weight of each, indicated that the differences are unlikely to be due to chemical fractionation. However, the heat of combustion of chitin is not accurately known, so more precise calculations and conclusions cannot be made at present.

(*iii*) *Endothermic region* (*ca.* 650–800°C). The combination of endothermic energy change (DTA) and rapid loss in weight (TG) at temperatures of *ca.* 650–800°C suggests that this region represents the evolution of carbon dioxide from decomposing calcium carbonate. At the end of region (ii) there was a period of no energy change in *Gammarus* and *Asellus* material, producing a 'plateau' in the DTA trace (Fig. 2) which was even more prominent in plant material. No such plateau occurred in crayfish material, where exothermic combustion occurred up to and possibly overlapped the onset of CO_2 evolution in region (iii). At this transition point for energy change (DTA) a loss of *ca.* 3–6% dry solids occurred (TG) which may represent the loss of organic components, CO_2, or both. For crayfish, therefore, total losses of CO_2 from the decomposition of $CaCO_3$ can only be accurately determined by the difference in CO_2 evolved from dry solids (60°C) and ash (800–1000°C), after treatment with acid.

(*iv*) *No energy change* (*ca.* 800–1000°C). This is a stable region with little or no change in weight of residual ash.

From the above results, we define dry solids as the mass remaining after drying to constant weight in an oven at 60°C. The organic component is the difference in weight between dry solids and material (inorganic

ash) present at the "plateau" or transition-point (DTA) temperature where decomposition of carbonate begins. To determine this temperature more accurately we used crayfish exuviae (E) heated at rising temperatures (20°C min^{-1}) up to predetermined levels, at which the temperature was held constant for *ca.* 18 hr.

TG–DTA at constant temperatures

The isothermal temperature was initially set at 550°C. Crayfish samples maintained at this temperature showed a pronounced slope on the TG trace, equivalent to a loss in dry mass of 0.34 ± 0.02 %hr^{-1} (due to evolution of CO_2). At 500°C a plateau on the TG trace occurred at 44–45% initial dry solids, whereas at 450°C a slight slope equivalent to 0.1% hr^{-1} loss in organic dry mass occurred on TG traces at about 50% dry solids. From these observations we conclude that the large exothermal DTA peak at *ca.* 460–550°C and the small exothermal peak at 550–650°C (Fig. 1), which occurred under constantly rising temperatures (20°C min^{-1}), represent an organic component which is difficult to oxidize due to its position in the matrix. In *Gammarus*, *Asellus* and plant material the DTA exothermal reactions had stopped at *ca.* 500–550°C under rising temperatures. Lack of material and time (on a hired instrument) prevented us from doing more than one isothermal experiment at 500°C with *Gammarus* exuviae, where no change in weight (TG) was observed after the initial rapid losses. We conclude that 500°C is the most appropriate temperature to maintain when ashing these materials. This should minimize the decomposition of carbonate and, if held at 500°C for periods of at least 18 hr, practically all of the organic components (in terms of percent dry mass) will be combusted in a 20 mg finely-ground sample. Larger samples may need ashing for longer periods.

Inorganic analyses

Results of chemical analysis of exuviae, whole-bodies and plant materials are given in Table 2, based on acid-digestion of dry solids. In addition, ashed material was analysed for sulphate in order to determine the amounts of inorganic sulphur. This was in all cases below the limits of detection, i.e. < 7 μg S mg^{-1} dry solids for exuviae and < 1.4 μg S mg^{-1} for whole-bodies and plants. Chloride (Table 2) was measured after carbonate fusion.

Summation of acidic and basic oxides (mass balance). Amounts of each element in dry solids were converted to the appropriate oxides and expressed as percentages (Table 3) of the total weight of ash. The latter was independently determined from the TG analysis. Chloride was included by representing it as HCl; allowance was made for the excess H_2O which this method introduces.

For *Gammarus* and *Asellus* the mass balance of measured elements accounts for all of the weight of ash from exuviae and whole-body material, within ±11%. A larger imbalance, up to 18%, was obtained for crayfish whole-body and exuviae (Table 3). Major sources of error in the mass balance table are thought to be: (1) analytical errors of *ca.* 1–2% for each determinand, (2) measurement of ash content, and (3) incomplete homogenization of powdered samples of dry solids. The latter were sub-sampled for determin-

Table 2. Analyses of animal and plant materials (μg mg^{-1} dry solids at 60°C)

Material	Ca	Mg	Na	K	Fe	Al	Total CO_2	Resid.* CO_2	Si	P	Cl	Ash†
Exuviae												
Crayfish (E)	182	1.96	7.07	4.02	< 0.1	< 4	139	16	3.3	6.64	13.1	510
Crayfish (EC)	270	1.97	7.15	5.42	< 0.1	< 4	173	11	0.1	6.50	12.5	550
G. pulex (E)	293	1.77	2.12	0.22	< 0.1	< 4	249	25	0.1	3.57	4.2	640
G. lacustris (E)	294	2.33	3.20	< 0.1	< 0.1	< 4	234	26	1.4	4.51	10.8	620
Asellus (E)	262	1.00	4.75	< 0.05	< 0.1	< 4	238	24	0.3	4.47	9.9	650
Whole body												
Crayfish (B)	107	1.07	11.20	7.39	< 0.03	< 1	85	-	1.0	4.74	16.8	317
Crayfish (C)	122	1.19	11.60	8.55	< 0.03	< 1	97	8	0.4	4.79	97.8	408
G. pulex (B)	97	1.60	7.27	7.50	< 0.03	< 1	68	4	0.5	2.94	8.5	220
G. lacustris (B)	89	1.41	5.63	7.81	0.32	< 1	71	8	1.7	3.49	6.7	215
Asellus (B)	124	1.15	7.24	8.94	< 0.03	< 1	92	4	2.2	3.18	5.8	275
Plants												
Nitella	18	4.57	3.30	18.80	5.46	1.6	0.1	< 0.1	12.0	0.71	30	125
2-day leached elm	36	1.65	1.45	3.05	0.93	0.3	0.6	< 0.1	15.1	0.16	< 1	90
14-day leached elm	32	1.40	0.86	0.48	1.25	1.1	<0.1	< 0.1	7.8	0.28	< 1	90

*CO_2 liberated by addition of acid to ash, after combustion at 1000°C.
†500°C.

Table 3. Inorganic mass balance for animal and plant materials dried at 60°C. The calculated mass for each oxide is expressed as a percentage of ash at 500°C (Table 2)

Material	CaO	MgO	Na_2O	K_2O	Fe_2O_3	Al_2O_3	CO_2	SiO_2	P_2O_5	HCl*	Δ**
Exuviae											
Crayfish (E)	50.0	0.6	1.9	0.9	0	0	27.3	1.4	3.0	1.9	- 13.0
Crayfish (EC)	68.7	0.6	1.8	1.2	0	0	31.5	0	2.7	1.7	+ 8.2
G. pulex (E)	64.1	0.5	0.4	0	0	0	38.9	0	1.2	0.5	+ 5.6
G. lacustris (E)	66.5	0.6	0.7	0	0	0	37.7	0.5	1.7	1.3	+ 9.0
Asellus (E)	56.5	0.3	1.0	0	0	0	36.6	0.1	1.6	1.1	- 2.8
Whole body											
Crayfish (B)	47.3	1.0	4.7	2.8	0	0	18.0	0.7	3.4	4.0	- 18.1
Crayfish (C)	41.9	0.9	3.8	2.5	0	0	23.7	0.2	2.7	18.0	- 6.3
G. pulex (B)	61.8	1.2	4.5	4.1	0	0	30.9	0.5	3.1	2.9	+ 9.0
G. lacustris (B)	58.1	1.1	3.5	4.4	0.2	0	33.0	1.7	3.7	2.3	+ 8.0
Asellus (B)	63.3	0.7	3.5	3.9	0	0	33.4	1.7	2.6	1.6	+ 10.7
Plants											
Nitella	20.2	6.1	3.6	18.1	6.2	2.4	0.1	20.6	1.3	18.1	- 3.3
2-day leached elm	56.0	3.0	2.2	4.1	1.5	0.6	0.7	36.0	0.4	0.5	+ 5.0
14-day leached elm	49.8	2.6	1.3	0.6	2.0	2.3	0	18.6	0.7	0.5	- 21.6

*Calculated value for HCl minus $\frac{1}{2}$ mole H_2O produced for each mole of H^+ balanced by Cl^-; expressed as % ash.
**$\Delta = (\Sigma$ constituents $- 100)$.

ation of different elements and the sub-samples may have contained differing proportions of organs and tissues from various parts of the body. These are known to vary in their chemical composition. Fragments of differently coloured materials were visible in samples after powdering by pestle and mortar. Measurements of the weight of ash remaining after combustion at 500°C have three sources of error: weighing, incomplete combustion of organic components (especially in crayfish material), and loss of inorganic components by volatilization. As is shown below, the latter is a major source of error due to loss of chloride when relatively large amounts of organic material are combusted at 500°C (Bock, 1979).

In whole-body samples of *G. pulex*, 36% of the extractable chloride and 18% of the extractable sodium were lost when dry solids were ashed at 500°C (Table 4). Corresponding losses for *G. lacustris* were 24% (Cl) and 19% (Na), but there was no significant loss for whole-bodies of *Asellus*. For all three species the largest amounts of chloride were found in samples combusted at 970°C in the presence of sodium carbonate. This can be regarded as a measure of total chloride, as the presence of large quantities of sodium carbonate will greatly reduce losses by volatilization but should solubilize all the chlorine that is present. Hence the extra amounts of chloride, relative to chloride extracted from the dry solids (Table 4), are minimum estimates for ions that are tightly bound within large organic molecules and are not determined by cold acid-extraction techniques when these are applied to dry solids.

We conclude that the ash contents given in Table 2 (last column) are underestimated, at least for *Gammarus* materials, due to loss of sodium and chloride. This produces an apparent 'excess' of inorganic components relative to ash, in the mass balance (last column, Table 3). We do not know why there is a large imbalance in the results for some crayfish materials but not in others (Table 3). The imbalance (−21.6%) for 14-day leached elm leaves may be partly attributed to a large error in determining the very small ash content.

Ionic balance

By making some assumptions about the speciation of particular elements, the sums of cations and anions were calculated and compared (Table 5). Results for exuviae of *Gammarus* and *Asellus* are remarkably similar; total anions balance 76–86% of total cations. The discrepancy is a little larger in whole-body material, where 67–77% of cations were balanced by the measured anions. Results for crayfish were more variable and there is a very large anion deficit in leached elm leaves, especially after leaching for at least 14 days, when approximately half of the total silicon had leached out.

Table 4. Dry weights and amounts of sodium and chloride (±95% confidence limits) in whole-bodies of *Gammarus* and *Asellus* after exposure to different temperatures. Results are based on three (*Asellus*) or five samples, each comprised of 30–50 animals

		Temp. (°C)	*G. pulex*	*G. lacustris*	*Asellus*
(a)	Dry solids (mg)	60	25.2 ± 0.3	25.2 ± 0.5	25.2 ± 0.5
(b)	Ash (mg)	500	6.1 ± 0.5	4.4 ± 0.3	6.6 ± 0.2
(c)	Loss of dry solids (%)	-	76	83	74
(d)	Na in dry solids (µg mg^{-1})	60	6.18 ± 0.13	5.57 ± 0.12	5.11 ± 0.26
(e)†	Na in ash (µg mg^{-1})	500	5.06 ± 0.11***	4.63 ± 0.50***	4.97 ± 0.69
(f)	Cl in dry solids (µg mg^{-1})	60	8.53 ± 0.80	6.72 ± 0.36	5.77 ± 0.67
(g)†	Cl in ash (µg mg^{-1})	500	5.42 ± 0.59***	5.14 ± 0.44***	5.74 ± 0.31
(h)‡	Cl in fused ash (µg mg^{-1})	970	9.68 ± 0.47**	8.29 ± 1.15**	6.95 ± 1.04*

†(e) or (g) Significantly lower than (d) or (f) respectively.
‡(h) Significantly higher than (f); analyses on (h) after fusion at red heat with Na_2CO_3.
Asterisks show significance levels of 5% (one), 1% (two) and 0.1% (three).

Composition of exuviae in G. pulex

The dry weight of exuviae from individuals of *G. pulex* is related to the dry weight of the whole animal at the start of the next intermoult stage (Fig. 3). Exuviae represent 38–41% of body dry weight in small animals (1–2 mg dry wt) and 35–36% in larger animals (5–10 mg body dry wt). The relative amounts of each element in the exuviae were calculated by assuming that 36% of the amounts found in whole-body samples would occur in the exuviae if the elements were distributed equally throughout the entire animal. Comparison of calculated and observed amounts for exuviae (Table 6) showed that, as expected, calcium and carbonate are concentrated *ca.* 10-fold in the exuviae, and to a much smaller extent so are magnesium and phosphate. In contrast, potassium and silicate apparently are present only in very small proportions in the exuviae.

DISCUSSION

Our investigations were necessarily very limited in scope and time, but they show that thermogravimetric and differential thermal analysis is a very useful tool for examining the effects of temperature on the decomposition of a variety of components in biological materials. When linked to a gas chromatograph, the analysis of gases as they are evolved during combustion would provide a very detailed picture of events and help to resolve some of the questions that are still outstanding with regard to the exact composition of some chemical components.

The loss of *ca.* 5% dry wt between 60 and 100°C (TG; Figs 1 and 2) confirms the well-known fact that the dry masses of organic materials decrease as the temperatures used for drying are increased, due to losses from residual chemically-bound water and volatilization of organic components, especially fats (Lovegrove, 1962, 1966; Grodzinski *et al.*, 1975). The definition of dry solids is thus specifically related to the manner in which materials are treated, particularly with regard to air temperature and pressure. Our results from DTA (Figs 1 and 2) also confirm that, although an apparently 'structured' inorganic ash is obtained at 500°C, this is the best temperature to use for complete (or near-complete) combustion of organic components whilst minimizing the loss of CO_2 from decomposing carbonates. Apart from loss of carbonate, it is usually stated in the literature that other major inorganic components begin to volatilize at *ca.* 550°C, e.g. sodium and potassium (Grove *et al.*, 1961; Grodzinski *et al.*, 1975). Because variations of ±50°C can occur in different parts of a muffle furnace it is important to ensure (using thermocouples) that materials are placed in the appropriate part of the furnace. Even so, with heavily calcified crustaceans, e.g. decapods, some decomposition of carbonates may occur unless the temperature is very accurately controlled. Moreover, our results for analyses of sodium and chloride after exposing whole-bodies to different temperatures (Table 4) indicate that in some instances, e.g. *Gammarus*, significant losses of these ions can occur when materials are ashed at 500°C. Thus, even when this temperature is used, the total mass of inorganic components may be underestimated and that of organic components will usually be overestimated. In our study the errors from this source were *ca.* 5–10%.

Calcium is the dominant cation in both whole-body and exuviae materials and much of it probably occurs as carbonate (Tables 2, 3 and 5). X-ray diffraction

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Table 5. Ion balance for animal and plant materials (μE g^{-1} dry solids at 60°C)

	Ca^{2+}	Mg^{2+}	Na^{+}	K^{+}	Fe^{3+}	Al^{3+}	Total CO_3^{2-}	SiO_3^{2-}	PO_4^{3-}	Cl^{-}	Σcations(a)	Σanions(b)	[(b)/(a)].100	Residual CO_3^{2-}
Exuviae														
Crayfish (E)	9100	161	307	103	0	0	6318	236	645	369	9671	7568	78	727
Crayfish (EC)	13500	162	311	139	0	0	7864	7	631	353	14112	8855	63	500
G. pulex (E)	14650	146	92	6	0	0	11318	7	347	119	14894	11854	80	1136
G. lacustris (E)	14700	192	139	0	0	0	10636	100	438	304	15031	11478	76	1182
Asellus (E)	13100	82	207	0	0	0	10818	21	434	280	13389	11553	86	1091
Whole body														
Crayfish (B)	5350	88	487	189	0	0	3863	71	460	474	6114	4868	80	-
Crayfish (C)	6100	98	504	219	0	0	4409	29	465	2756	6921	7659	111	364
G. pulex (B)	4850	132	316	192	0	0	3091	36	285	239	5490	3651	67	382
G. lacustris (B)	4450	116	245	200	17	0	3227	121	339	189	5028	3876	77	364
Asellus (B)	6200	95	315	229	0	0	4182	157	309	163	6839	4811	70	182
Plants														
Nitella	900	376	143	482	292	178	5	857	69	849	2371	1780	75	0
2-day leached elm	1800	136	63	78	50	33	27	1079	16	0	2160	1122	52	0
14-day leached elm	1600	115	37	12	67	122	0	557	27	0	1953	584	30	0

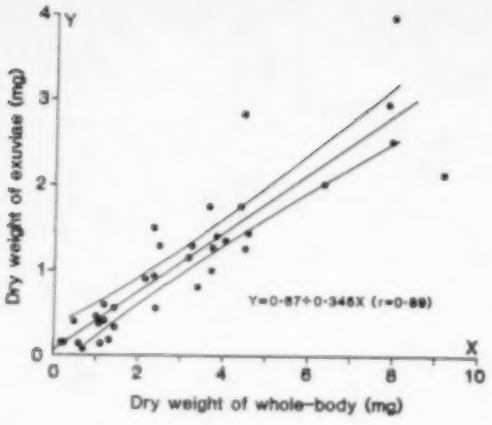

Fig. 3. Dry weight of exuviae plotted against total body dry weight of *G. pulex* after moulting. The regression line and 95% confidence limits are shown.

analysis (unpublished) showed major peaks due to calcite and, possibly, traces of aluminosilicates. However, the presence of residual carbonate after heating to 1000°C suggests that some of it occurs as sodium carbonate in exuviae of *Gammarus* and *Asellus*, and possibly also as potassium carbonate in exuviae of crayfish. Unlike calcium and magnesium carbonates, those of sodium and potassium do not volatilize or decompose at temperatures below 1200°C. In all of the materials examined by us, the amounts of calcium (Y, μE mg^{-1} dry solids) always exceeded those of carbonate (X, μE mg^{-1} dry solids) and a linear regression of Y on X for whole-bodies and exuviae gave the equation:

$$Y = 1.21\,X + 1.42;\ r = 0.97.$$

We therefore conclude that up to 80% of the calcium may be present as carbonate. The remainder is not balanced by any particular anion but by different anions in different materials. The overall deficits in total anions were mostly 14–37% of total cations. These deficits (Table 5) presumably indicate the fractions that consist of negatively charged organic acids in living materials. Organic acids, especially amino acids, have a major role in maintaining ionic balance in tissue cells and they occur in relatively large quantities in crustaceans (e.g. Robertson, 1957; Shaw, 1958; Sutcliffe, 1963; Schoffeniels and Gilles, 1970). The cuticle contains numerous amino acids in arthropodin and sclerotin (Welinder, 1974, 1975a,b; Vigh and Dendinger, 1982).

The exuviae of *Gammarus*, *Asellus* and crayfish all contained relatively large amounts of phosphate. This is probably balanced by calcium (and magnesium ?) ions and, in crayfish exuviae, perhaps also by potassium; the exuviae of *Gammarus* and *Asellus* contained only traces of potassium (Tables 5 and 6). Hydroxyapatite, $Ca(OH)_2 \cdot 3Ca(PO_4)_2$, is probably the basic form of phosphate in crustacean skeletons (Needham, 1954). If all of the phosphate in exuviae occurs in this form it would be balanced by *ca.* 2.4–3.0% of the calcium in exuviae of *G. pulex* and *G. lacustris*, 3.3% in *Asellus* and 4.7–7.1% in crayfish. Thus only a very small proportion of the calcium lost in exuviae of *G. pulex* and *G. lacustris* is in the form of

Table 6. Major components in whole-bodies and exuviae (μE g^{-1}) and concentration factors for exuviae of *G. pulex*

Component	Whole-body (1, Observed)	Exuviae (2, Observed)	Exuviae (3, Calculated)*	Concentration factor in exuviae (2/3)
Ca	4850	14650	1746	8.4
Mg	132	146	48	3.0
Na	316	92	114	0.8
K	192	6	69	0.1
Total CO_3	3091	11318	1113	10.2
Residual CO_3	182	1136	66	17.2
Cl	239	119	86	1.4
Si	36	7	13	0.5
PO_4	285	347	103	3.4
Organic†	780	360	280	1.3

*Assuming 36% of whole-body values (see the text).
†Expressed as μg mg^{-1} dry solids.

hydroxyapatite. Huner *et al.* (1979) describe postmoult deposition of phosphate in the cuticle of a marine shrimp.

There are very few analyses for detailed comparison with our results. Concentrations of the minor elements in these freshwater crustaceans are similar to, but slightly lower than, values given by Clarke and Wheeler (1922) for some marine Crustacea. Estimates for carbonate also fall within their range of values but calcium, the major inorganic component, is up to 13% higher. The calcium content of *G. pulex* exuviae (Table 2) agrees very well with the value of 300 μg mg^{-1} dry wt given by Graf (1968), and calcium in crayfish exuviae agrees with values given by Chaisemartin (1962, 1964). Sodium concentrations in whole-bodies of *G. pulex* and *Asellus* are in accord with previous determinations (Sutcliffe, 1971, 1974); so is the potassium content of *G. pulex* but chloride values are slightly lower for *G. pulex* and higher for *Asellus*. Calcium in the whole-bodies of freshwater crustaceans has been more extensively measured. Our values for *G. pulex* (Table 2) are similar to the range of 30–124 μg mg^{-1} dry wt obtained by Vincent (1963), Chaisemartin (1964), Graf (1964, 1968) and Wright (1980b). Nevertheless the internal concentrations of all ions may be expected to vary, depending on the ionic composition of the external water and the stage of growth of the experimental animal, especially the phase of intermoult or ecdysis for each individual. It is also evident (Table 4) that different extraction techniques may give relatively large differences for analyses of inorganic ions. Hitherto this important fact has received little attention. Brine and Austin (1981) report on a similar problem with respect to the organic composition of chitin in crustacean cuticle.

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AN EXPLANATION FOR THE FOLDING FIBERS IN PROBOSCIS RETRACTOR MUSCLES OF *PHASCOLOPSIS* (=*GOLFINGIA*) *GOULDI*—I. CORRELATION BETWEEN LENGTH–TENSION DATA AND STRUCTURE IN LIVING MUSCLE

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Abstract—1. Fiber diameters and fold angles were measured at various muscle lengths at rest and during contraction.
2. The length–tension relationship was bimodal, with tension peaks at 1.0 and 1.67 L/L_0.
3. Fiber folding occurred during contraction at lengths $<1.3\ L_0$.
4. Diameter increased in folding cells during contraction only at lengths $>1.15\ L/L_0$.
5. We suggest that two cell types produce the bimodal length–tension curve.
6. We also suggest that folding cells produce force at longer muscle lengths where they are unfolded, but they are passively folded by a second cell type which produces force at shorter muscle lengths.

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INTRODUCTION

The proboscis retractor muscles of *Phascolopis* (=*Golfingia*) *gouldi* have long been classified as smooth or non-striated muscles. However, Prosser (1967) reported that during contraction, tranverse bands were evident under polarized light. Light microscopy of fixed preparations showed no evidence of the classical crossbands of striated muscle. Instead, the banding was shown to be due to folding, in register, of individual fibers, producing a "herringboned" appearance (Prosser, 1967). It is interesting that several earlier studies of the influence of length and contractile state upon birefringence in the proboscis retractor had been made (Bozler and Cottrell, 1937; Fischer, 1936, 1938, 1944) and it was concluded that the birefringence changes during stretch were consistent with a change in orientation of birefringent "micelles" (Bozler and Cottrell, 1937; Fischer, 1936) but neither the transverse banding pattern nor the folding cells were described in these earlier studies.

Matsumoto and Abbott (1968) made the first study of this muscle which combined physical measurements and cell structure. They showed that folding fibers in the proboscis retractor muscle were responsible for the birefringent banding by viewing the muscle first with polarized light at a low magnification and then fixing the tissue and viewing thick sections cut for electron microscopy with polarization optics at a magnification sufficient to discern individual fibers. They also reported a bimodal length–tension curve for this muscle, while other muscles have a unimodal length–tension curve. (For a discussion of the relationship between structure and the shape of the length–tension curve, see Gordon *et al.* (1966) and Murphy (1976)). Matsumoto and Abbott (1968) suggested two populations of cells could account for the bimodal length–tension curve. A population of straight fibers interspersed among the folded fibers could also produce the necessary force to fold the other population of muscle fibers. Otherwise, force would have to be produced by the folding cells while folding, a seeming contradiction to the sliding-filament hypothesis of contraction (Huxley, 1957; Huxley and Hanson, 1954; Huxley and Niedergerke, 1954). Ernst (1970) provided support for the two-cell hypothesis advanced by Matsumoto and Abbott by using electron microscopy to show that two types of cells did exist, one of which folded (large cells) and one of which remained straight (small cells). However, the structural evidence was almost entirely from fixed material, and there was no direct proof that the folding of the large cells was due to contraction of the small cells.

We decided to test the hypothesis of Matsumoto and Abbott (1968) by using observations of living material in addition to fixed material. Our first set of experiments utilized isolated cells prepared by collagenase digestion (Abercrombie and Bagby, 1975). We found that isolated cells sometimes showed folded configurations at rest, but upon contraction the folding disappeared, sometimes reappearing when the cell subsequently relaxed. Although these experiments were instructive in showing that active folding did not seem to be a property of individual cells, the mechanism responsible for folding can apparently only be studied under conditions where folding occurs during contraction, i.e. in the whole muscle.

In the present study we continued our testing of the hypothesis that the folding cells fold passively due to

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activity of other cells. We sought to answer two main questions:

1. Does folding occur in cells of living contracting whole muscle? (This had only been inferred from birefringent banding patterns in living muscle and histology of fixed muscle.)
2. If cells do fold, is the force for folding produced by the folding cells themselves?

A system was developed where physical measurements (isometric tetanic tension and length) of living muscles could be recorded while photographing either the birefringent banding or the individual fiber configuration in the proboscis retractor muscle, utilizing polarization microscopy. The simultaneous recording of physical measurements and photographing the living muscle both at rest and during activity allowed, for the first time, direct evidence to be collected and analyzed as to the detailed relationship between the birefringent banding pattern of the proboscis retractor muscle, the configuration of the folding fibers, and the length–tension relationship. Brief accounts of some of our findings are found in earlier abstracts (Abercrombie, *et al.*, 1977; Abercrombie and Bagby, 1978).

The second paper (Abercrombie and Bagby, 1984) provides additional structural evidence from fixed cells taken from some of the muscles used in the present study.

MATERIALS AND METHODS

In all experiments, the ventral proboscis retractor muscles (VPRM) of *Phascolopsis* (= *Golfingia*) *gouldi* were used. The muscles were excised with scissors at points approximately 3 mm from their attachments on the body wall and at the esophageal–tentacular complex. One of the muscle pair was then surgically isolated and tied at both ends with surgical thread (Ethicon 00 silk). The VPRM was then allowed to equilibrate for approximately 1/2 hr in oxygenated Sipunculid Ringer at room temperature (23°C) and pH 7.8, which contained osmotically balanced calcium-free and magnesium-free "Instant Ocean"* sea water plus: 11.67 mM $CaCl_2$, 33.02 mM $MgCl_2$ and 0.22 mM glucose. Sipunculid Ringer was designed so that there would be amounts of calcium, magnesium and glucose in the Ringer solution that mimic the coelomic fluid of *P. gouldi*. Foster (1974) reported that the above values for calcium, magnesium and glucose along with standard sea salts are equivalent to the coelomic fluid of *P. gouldi*.

The experimental chamber was designed so that the same precise area of the muscle could be photographed, utilizing polarization optics, at various points in the length–tension relationship. The chamber consisted of a 13 × 5.5 × 1.5 cm lucite chamber in which twelve Ag-$AgCl_2$ electrodes were embedded in agar 1 cm apart (except at the viewing area where electrodes were embedded 0.5 cm apart) transversely with respect to the muscle axis. The Ag-$AgCl_2$ electrodes were coated with 1% agar in Sipunculid Ringer prior to each experiment. The chamber was attached to the substage, which was in turn attached to the stage of a Nikon inverted polarizing microscope for the low magnification length–tension study, or to the stage of a Nikon SUKE II microscope with polarizing optics for the high magnification length–tension study. Electrode leads were then connected to a Grass S44 stimulator and SIU5 stimulus-isolation unit, which produced a stimulus of 100 V, 5 msec duration, and 5 pulses per sec to tetanize the muscle during the measurements.

The muscle with attached surgical threads was placed horizontally in the chamber and immersed in Sipunculid Ringer. The surgical threads were passed through nylon-grommeted holes at opposite ends of the lucite chamber. The grommets were sufficiently tight to prevent leaking but the thread could pass smoothly. One thread was then tied to a spool on the substage, which served to take up slack or stretch the muscle. The thread at the opposite end of the preparation was tied to a hook portion of an extension post on a RCA 5734 force transducer tube (RCA Corporation, Camden, N.J.). The transducer tube was housed in a heat sink which was mounted on a sliding track on the substage. Thus, the same precise portion of the muscle could be kept in view while stretch was applied to lengthen the muscle.

The transducer was calibrated by measuring its output in relation to length changes of a spring that had previously been calibrated by hanging weights from it. The 5734 was linear for forces from 0–10.0 g when the extension post extended 8.0 mm beyond the end of the plate shaft. Permanent records of isometric tetanic tension were made utilizing a Beckman Type RB Dynograph.

For photographic purposes, the muscle was barely covered with Sipunculid Ringer when records were being made. Between measurements it was deeply covered by the Ringer. Photographic records of the configuration of the living fibers were made utilizing Kodak Tri-X 35mm film (ASA 400) and a Nikon Standard Microflex PFM camera attachment mounted on either the Nikon inverted microscope or the Nikon SUKE II microscope.

The length–tension relationship was generated from the data collected from the physical measurements. These various lengths (L) and tensions (P) were then normalized (L/L_0 and P/P_0 respectively, where P_0 = maximum tension recorded and L_0 = the length of the muscle where P_0 was recorded) to correct for differences in muscle sizes and maximum forces between different experiments. Several other measurable parameters were compared to the normalized length (L/L_0) once the length–tension relationship had been established.

RESULTS

Does folding occur in cells of living contracting whole muscle?

The ventral proboscis retractor muscle (VPRM) of *P. gouldi* exhibits an ordered birefringent banding pattern over a wide range of lengths at low magnification. The individual fibers are responsible for the birefringent banding pattern due to the folding of the fibers and/or abrupt changes of the angle which the birefringent elements make with the plane of the polarized light. Thus on either side of a fold,one side will exhibit more brilliance than the other due to its birefringent elements being more favorably oriented with respect to the plane of polarized light. Alternate light and dark bands are thus produced, for the most part in register across the whole muscle. The interval between bands coincides exactly with the interval between folds, as has been shown for the whole muscle and subsequent fixed preparations by Matsumoto and Abbott (1968).

Figure 1 is representative of the type of structural data collected at high magnification. The line drawing (Fig. 2) explains the parameters which were to be measured. One can clearly see that the fiber band width (B.W.) which is defined as the distance from one peak of the fiber orientation to the next peak of

*Available commercially from "Instant Ocean", Aquarium Systems Inc., Wickliffe, OH, USA.

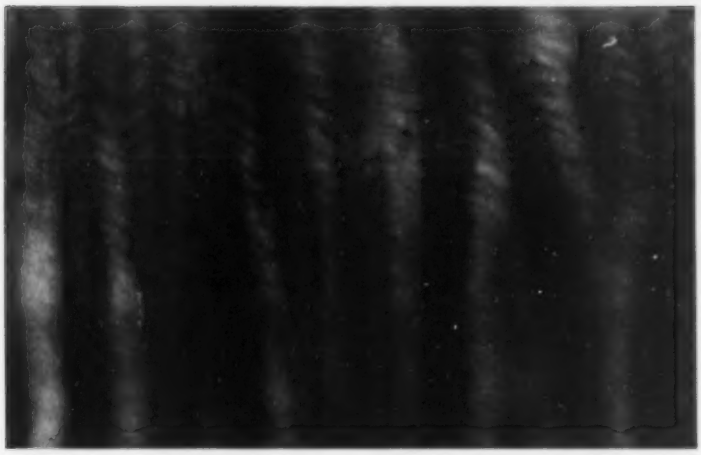

Fig. 1. A light micrograph with high-power polarizing optics showing the relationship between folds of individual fibers and the birefringent bands width (B.B.W.)—the distance from the beginning of one bright area to the beginning of the next bright area.

the same orientation is usually identical to the birefringent band width (B.B.W.) which is defined as the distance from the beginning of one bright area to the beginning of the next bright area. The correlation between birefringent band width and fiber folding can be observed from the portion of the photomicrograph which is in sharp focus, showing the orientation of the fibers in register, and the portion of the photomicrograph which is partially out of focus, showing the birefringent band width. The fold angle θ defined as the angle the fiber makes with the longitudinal axis of the muscle, the segment lengths (S.L.) defined as the length along the fiber from bend to bend, and the diameter (D) of the segment, may also be determined from such a micrograph.

Figure 3 illustrates the relationship between the length of the muscle and the birefringent band width in a typical experiment. The muscle was stretched in 0.5 cm increments and it can be seen that the B.B.W. changes regularly with the change in length. At each 0.5 cm increment of length change the muscle was stimulated at 5 c/sec, an isometric tetanic contraction was recorded and a photographic record of the muscle was made. From these initial data, the length of the muscle (L) and tension (P) were normalized to obtain L/L_0 and P/P_0 as explained previously. A bimodal length–tension curve was obtained as shown in Fig. 4a. Two active tension peaks are apparent, one at 1.0 L/L_0 and the other at 1.67 L/L_0. The peak at 1.0 L/L_0 was the maximum active tension (P_0). At the second peak, the active tension (P/P_0) was 0.7. Passive tension was first recorded at 1.45 L/L_0 and then rose exponentially with increasing length.

Figure 4b shows the relationship of birefringent band width (B.B.W.) to muscle length on the same scale as the length–tension diagram (Fig. 4a), with measurements of B.B.W. both at rest and during contraction. The B.B.W. measurements increase almost linearly from a minimum of about 20 μm to a maximum of about 60 μm over the length range of 0.5–1.7 L/L_0. A plateau in B.B.W. measurements begins at about 2.0 L/L_0 and there is no further increase even with increases in muscle length. At lengths between 0.5 and 1.5 L/L_0 there is a small but consistent decrease in B.B.W. of about 5 μm when the muscle is stimulated to contract. The slight decrease in B.B.W. between rest and activity is possible due to the slight shortening of the muscle during contraction, which is allowed by the compliance of the recording lever of the transducer. We attribute these decreases in B.B.W. to progressive folding at shorter muscle lengths and to increased folding during active contraction. This is borne out by Fig. 5 which shows the relationship between fold angle and muscle length. The angle which the cell makes with the muscle axis increases as the muscle length is decreased below 1.3 L/L_0. Also, the angle increases during active contraction at lengths below 1.3 L/L_0.

From these observations one can conclude that folding of some cells is a phenomenon which does occur in living whole muscles, and the extent of folding is correlated with the relative muscle length. Furthermore, at least in whole muscles, folding persists during contraction and the folded cells become more folded during active force production.

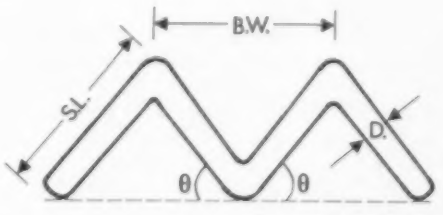

Fig. 2. A line drawing illustrating the parameters measured from micrographs. B.W. is fiber band width, S.L. is segment length, θ is the fold angle of folded cells, and D is the diameter of the segment.

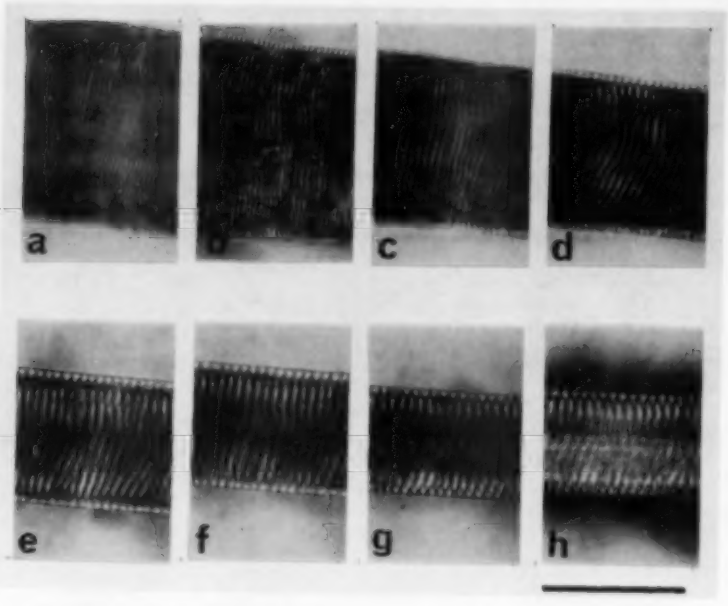

Fig. 3. Low-power micrographs of a ventral proboscis retractor muscle as the muscle is stretched from 2.5 cm to 6.0 cm in 0.5 cm increments (a–h, respectively). Note that birefringent band width increases and the muscle decreases in diameter as the muscle is stretched from 2.5 to 6.0 cm. Dimension bar equals 1.0 mm.

Does folding of cells occur over the entire range of muscle lengths?

From Fig. 4b, it can be seen that B.B.W. changes almost linearly between 0.5 and about 1.67 L/L_0. Since B.B.W. usually coincides with the periodicity of bends in the cells, this would seem to indicate that folding occurs between 0.5 and 1.67 L/L_0. However, from Fig. 5 it can be seen that fold angle reaches 0° at about 1.25 L/L_0, so that the increased B.B.W. between 1.25 L/L_0 and 2.0 L/L_0 *cannot* be due to a continued flattening of the folds in the muscle.

It is possible that some internal arrangement of birefringent material continues to give rise to the B.B.W. even at lengths where the cell is not folded. It is also possible that there is some connective tissue element that coincides with the position of the fold and indeed may be responsible for the folds being in register across the muscle, which may provide a repeating birefringent pattern in this length domain. Whatever causes the continued increase in B.B.W. from 1.3 L/L_0 to 2.0 L/L_0, it is clear from Fig. 5 that folding is a discontinuous function of length. Folding of muscle cells occurs only between 0.5 L/L_0 and 1.3 L/L_0. The region of the length–tension relationship where folding occurs is therefore under the first tension peak, and the length domain where the cells remain straight is under the second tension peak (compare Fig. 5 with Fig. 4a).

It can be seen that the amount of change in folding produced by active contraction of the muscle is dependent upon muscle length. As the muscle is lengthened between 0.5 L/L_0 and 1.3 L/L_0 there is progressively less difference between fold angles at rest and during activity, with a convergence of rest and activity values at about 1.3 L/L_0.

We have no explicit explanation for why the B.B.W. reaches a maximum at 2.0 L/L_0 and does not increase as the muscle is stretched further (see Fig. 4b), but it could be that cells tear apart at these lengths leading to greater muscle length without changing cell lengths.

Is the force for folding produced by the folding cells?

One cannot easily measure tension in the folding cells alone to ascertain whether they are actively producing tension while folding, but one can make inferences from other measurements. For example one could measure segment length and diameter in the folded cells. If segment length decreased while diameter increased, one could be reasonably sure that force was being actively produced. We initially attempted to measure both segment length and diameter, but we abandoned segment length measurement when it was discovered that the folds occurred in the vertical as well as horizontal plane, making accurate measurements impossible. However, accurate diameter measurements could be made, and the relationship between segment diameter and muscle length in both resting and active muscles is shown in Fig. 6. It is interesting that there was no measurable difference in diameters of resting or active cells at lengths below 1.15 L/L_0, while a consistent increase in diameter occurred during activity at lengths greater than 1.15 L/L_0. Thus the folding cells appeared to be actively shortening and consequently increased in diameter at lengths greater than 1.15L/L_0, but at shorter lengths where the cells were noticeably folded they did not appear to undergo dimensional changes associated with contraction.

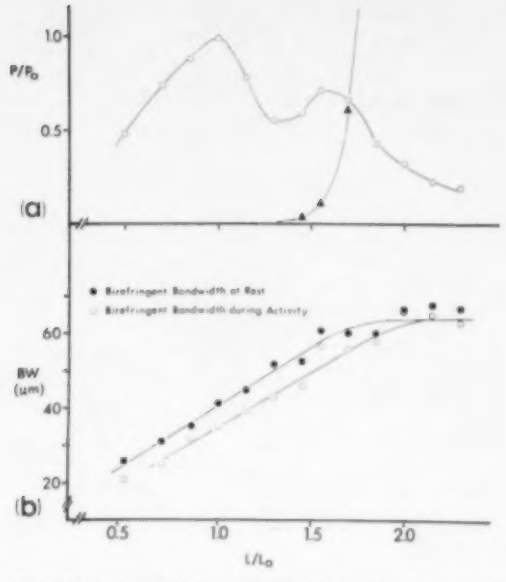

Fig. 4. The normalized length–tension relationship (a) and the length–birefringent band width relationship (b) are plotted on the same normalized length scale so that the two relationships may be compared. (a) The length scale (L/L_0) and tension scale (P/P_0) have been normalized to allow the use of data from five muscles where absolute values of length and tension varied. L = length, L_0 = length at which maximum tetanic tension was obtained. P = tetanic tension, P_0 = maximum tetanic tension. Active tension (○) and passive tension (▲) are shown. Note the bimodal nature of the relationship, with L_0 at 1.0 L/L_0 and a second smaller peak at 1.67 L/L_0. Passive tension is first recorded at 1.45 L/L_0 and rises exponentially at greater lengths. (b) Birefringent bandwidth (B.B.W.) was determined from micrographs such as those shown in Fig. 3 and plotted on the same length scale as the length–tension relationship. Band width was measured at rest (●) and during tetanic tension (○). There is a linear relationship between length and band width at length values between 0.5 and 1.5 L/L_0. Note the consistent decrease in band width from 0.5 L/L_0 to 1.5 L/L_0 when the muscle is stimulated. This coincides with lengths where the first tension peak predominates. At lengths beyond 1.5 L/L_0, resting and active values converge and both tend to plateau beyond 2.0 L/L_0. This coincides with lengths where passive tension becomes significant and where the second tension peak predominates. Data points are the means from five muscles.

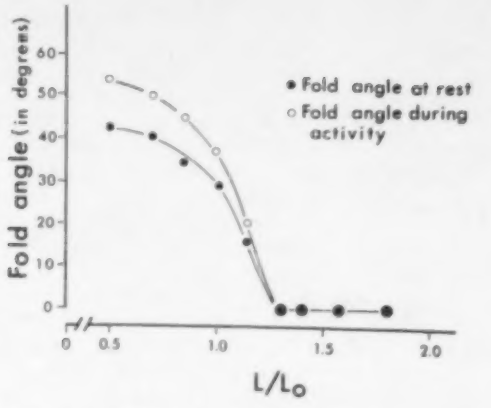

Fig. 5. The relationship between the normalized length of the muscle (L/L_0) and the fold angle (θ in Fig. 2) of the folded cell at rest (●) and during tetanic tension (○) is shown. Note the increase in fold angle during activity between 0.5 and 1.3 L/L_0 and that the amount of increase in fold angle during activity decreases as muscle length increases. Folding occurs only in length domain where the first tension peak is obtained. Data points are means from eight muscles.

Therefore we conclude that the force for folding is *not* provided by the folding cells.

DISCUSSION

The length–tension curve of skeletal muscles is a unimodal curve (Gordon *et al.*, 1966). For some vertebrate smooth muscles (Åberg and Axelsson, 1965; Meiss *et al.*, 1974; Murphy, 1976; Uvelius and Gabella, 1980) and at least one invertebrate smooth muscle (Ishii and Takahashi, 1982), this curve tends to be flattened, and it may extend over a greater range of muscle lengths, but only unimodal curves have been reported (Åberg and Axelsson, 1965; Ishii and Takahashi, 1982; Murphy, 1976, 1980; Meiss, 1974; Uvelius and Gabella, 1980). Matsumoto and Abbott (1968) reported a bimodal length–tension relationship for the proboscis retractor muscle of *Phascolopis* (=*Golfingia*) *gouldi* and we have confirmed their findings in the present investigation. The length–tension relationship and the correlated structural changes at various lengths have provided strong evidence for the sliding filament mechanism in skeletal muscles, with the maximal tetanic tension occurring when there is "optimal" overlap between thick and thin filaments (Gordon *et al.*, 1966). A bimodal

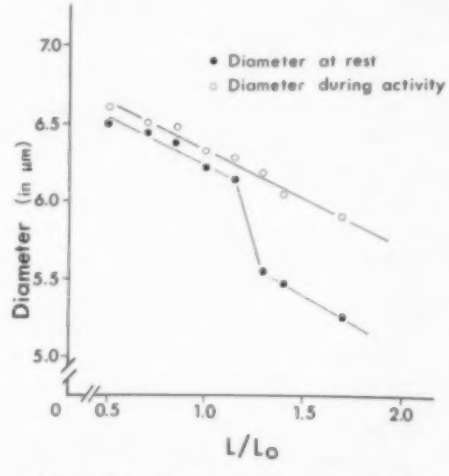

Fig. 6. The relationship between the normalized muscle length (L/L_0) and the diameter of the folding cell at rest (●) and during tetanic tension (○) is shown. Tension production in the muscle does not result in an increase in fiber diameter between 0.5 and 1.15 L/L_0, but the fibers do increase in diameter when tension is produced at muscle lengths greater than 1.15 L/L_0. Data points are means from eight muscles.

length–tension curve would imply *two* lengths at which there is optimal overlap. For this to occur in a single cell would imply a much different arrangement of thick and thin filaments.

The muscles of sipunculids are the only ones which have been reported to fold during contraction. The present investigation has shown that the folding is not an artifact of fixation and it is not due to elongation of cells during relaxation as in single cells (Abercrombie and Bagby, 1975), but that folding of cells occurs in living whole muscles while the muscles are producing force. The sliding filament mechanism (Huxley, 1957; Huxley and Hanson, 1954; Huxley and Niedergerke, 1954) does not provide a means of producing force while folding. In fact, the *straightening* of bent myofibrils in skeletal muscle has been used as a means of detecting activity (Gonzalez-Serratos, 1971). For the folding fibers to be producing the force necessary for folding, one would have to invoke some mechanism not accounted for by the sliding filament mechanism.

Both the bimodal length–tension relationship and the folding are most easily explained by the two-cell hypothesis advanced by Matsumoto and Abbott (1968). We have shown that the folding cell produces force only in the length domain of 1.15 to 2.0 L/L_0 (Fig. 6), which coincides with the area under the second tension peak (Fig. 4a), while folding occurs only from 0.5 to 1.3 L/L_0 (Fig. 5), the area under the first peak (Fig. 4a). Therefore, we conclude that the folding cells are responsible for a unimodal length–tension curve with its peak coinciding with the second peak, and suggest that a second population of cells produces a unimodal length–tension curve with its peak coinciding with the first tension peak. Furthermore, we suggest that the force produced by the second population of cells is the force causing the cells to fold, and that the second population of cells does not fold. It is possible that there might be two populations of the same cell type which differ only in their relative cell lengths at a given muscle length. This type of arrangement provides an attractive explanation for smooth muscles whose length–tension curve is sometimes flattened (Åberg and Axelsson, 1965; Ishii and Takahashi, 1982; Meiss *et al.*, 1974; Murphy, 1976; Uvelius and Gabella, 1980). In this case the flattening would be due to different cells existing at several relative cell lengths at any particular muscle length, resulting in different cells having their tension peaks at different muscle lengths and a composite curve with a flattened peak. However, two cell types—large cells and small cells—have been described for *Phascolopsis (= Golfingia) gouldii* retractor muscles (Ernst, 1970), and it seems likely that these two cell types are responsible for the two tension peaks. The paper which follows (Abercrombie and Bagby, 1984) utilizes scanning electron microscopy and light microscopy to measure parameters of cells from muscles fixed at particular points on the length–tension relationship. It provides additional evidence for small fibers being responsible for the first tension peak, for large fibers providing the second peak, and for the large fibers being passively folded by contraction of the small fibers.

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AN EXPLANATION FOR THE FOLDING FIBERS IN PROBOSCIS RETRACTOR MUSCLES OF *PHASCOLOPSIS* (=*GOLFINGIA*) *GOULDI*—II. STRUCTURAL EVIDENCE FROM SEM OF TEASED, GLYCERINATED MUSCLES AND LIGHT MICROSCOPY OF KOH-ISOLATED FIBERS

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Abstract—1. After length–tension analysis, muscles were fixed at known relative lengths (L/L_0).
2. SEM observations were made on teased glycerinated muscles.
3. Isolated cells were obtained by KOH maceration.
4. Large diameter fibers folded at L/L_0 values < 1.3, were straight at L/L_0 values ≥ 1.3 and were most active at L/L_0 values ≥ 1.0.
5. Small diameter fibers never folded and were most active at L/L_0 values < 1.5.
6. We conclude that small fibers are responsible for the tension peak at 1.0 L/L_0, large fibers for the peak at 1.67 L/L_0, in the bimodal length–tension relationship.
7. We conclude that large fibers fold passively due to active shortening of small fibers.

INTRODUCTION

It has been known since 1967 (Prosser, 1967) that the proboscis retractor muscles of *Phascolopsis* (=*Golfingia*) *gouldi* exhibited folded fibers in the contracted state. Despite studies since then, it has not yet been determined whether the force for folding comes from a separate straight fiber type. Matsumoto and Abbott (1968) reported a bimodal length–tension relationship and suggested that two populations of cells were responsible for the two tension peaks. Ernst (1970) characterized two cell types, the folding cell which had a larger diameter, and a smaller diameter cell which also contained paramyosin filaments. The existence of two distinct cell types has also been shown in the proboscis retractor of another sipunculid, *Sipunculus nudus* (Reger, 1964).

In the preceding paper (Abercrombie *et al.*, 1984) it was shown, for the first time, that the fibers folded in a contracting, whole, living muscle at relative muscle lengths below 1.3 L/L_0 and that the folding increased during contraction, eliminating the possibility that the folded configuration seen in fixed muscles was an artifact due to elongation of the large fibers in a muscle held at a shorter length. It was also found that the folding fibers did not increase their diameter when the whole muscle shortened at lengths below 1.15 L/L_0, showing that the folding fibers did not contract actively in the length domain where folding occurred. It was suggested that the folding of the fibers was a passive folding brought about by the active contraction of a second cell type, the "small" cell described by Ernst (1970). It was assumed that the second cell type remained straight during its active contraction. We also agreed with the hypothesis of Matsumoto and Abbott (1968) that two cell types were responsible for the two tension peaks in the length–tension relationship. However, the proof for the role of the second cell type could not be obtained with the methods used by Abercrombie *et al.* (1984), since a second cell type could not be clearly seen in the whole muscle.

Several methods have been used to study configurations of individual muscle cells. Striated muscles may be teased into individual fibers either in the living state, or with greater ease from glycerinated muscles, with few fine structural changes (Sjöstrand and Jagendorf–Elfvin, 1967). Fibers from *P. gouldi* proboscis retractor muscles have been satisfactorily prepared in this way (Matsumoto and Abbott, 1968). Most vertebrate smooth muscles have too much connective tissue to allow teasing, but enzymatic digestion of the connective tissue allows living smooth muscle cells to be isolated from several preparations (Bagby *et al.*, 1971, 1976; Bagby and Pepe, 1978; Momose and Gomi, 1977; Small, 1974). Living cells have also been prepared from *Phascolopsis* (=*Golfingia*) *gouldi* by using enzymes (Abercrombie and Bagby, 1975). However, one cannot be certain that the configurations seen in the living isolated cells are the same as when the cells were in the whole muscle. Cells frequently round up, shorten, or change their shape in other ways when freed from the mechanical restraints of the whole muscle. Cells may also be released from fixed tissue by using KOH to remove connective tissue (Cooke and Fay, 1972). This latter method has the advantage of allowing one to make mechanical measurements on the whole muscle prior to fixation and to preserve the structural configuration of the cells in the state at which the whole muscle was fixed.

The methods used in the present paper allowed us

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to see individual cell configurations of the two cell types while retaining the advantages of the length–tension measurement to establish relative muscle length. In this way the configuration of individual cells could be determined at specific points on the length–tension curve, allowing us to determine whether a particular cell type was folded or straight in a particular length domain. In the preceding paper (Abercrombie *et al.*, 1984) an increase in diameter of a living fiber during stimulation was used as a criterion for active contraction. In the present paper, fixing muscles at different relative muscle lengths (L/L_0) either in the relaxed or contracted state, followed by KOH maceration to obtain individual cells, allowed us to use fiber diameter measurements to determine the length ranges where each cell type was capable of active contraction. We believe that the preceding paper (Abercrombie *et al.*, 1984) and the present paper, by showing that the two cell types operate in different length domains, provide the best explanation for the anomalous behaviour of the whole muscle.

MATERIALS AND METHODS

Scanning electron microscopy (SEM)

In all experiments, the ventral proboscis retractor muscles (VPRM) of *Phascolopsis* (= *Golfingia*) *gouldi* were used. The muscles were fixed at their respective lengths, after determining the length–tension relationship (Abercrombie *et al.*, 1984), by tying each specific muscle at the desired length on an applicator stick. The muscles were allowed to equilibrate for $\frac{1}{2}$ hr in artificial seawater (Abercrombie *et al.*, 1984) (pH 7.8) at 0–4°C. The muscles were then placed in a glycerination solution consisting of 50% glycerol, 50% Standard Salt Solution (Huxley, 1963) (100 mM KCl, 10 mM phosphate buffer, 1 mM $MgCl_2$ pH 7.0) for 24 hr at 5°C. The glycerination solution was then exchanged for fresh glycerination solution and stored at −20°C at least one month prior to use. At that time, the preparations were placed in fresh Standard Salt Solution overnight at 5°C to remove the glycerol, then fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 6.5), 0.8 M sucrose and 5% DMSO for 1 hr at 0–4°C. This fixation method was the best of several at different pH values and temperatures. The preparations were then teased apart gently so individual fibers were exposed. Following teasing, the specimens were dehydrated in a series of acetone concentrations and critical-point-dried utilizing Bomar Critical Point Apparatus. They were then mounted on scanning electron microscope pedestals using DAG 154 (Ted Pella, Inc.), then carbon-coated, and subsequently gold-coated utilizing a Denton DV 515 Evaporator. The specimens were viewed with an ETEC Scanning Electron Microscope. Photographs were taken with Polaroid Type 150 N/P at initial magnifications of 170×–600×.

KOH cell isolation

In all experiments VPRM of *Phascolopsis* (= *Golfingia*) *gouldi* were used. The length–tension relationship was determined for each muscle used (Abercrombie *et al.*, 1984). Muscles were then tied to wooden applicator sticks to obtain lengths of 0.5, 1.0, 1.3, 1.5 or 1.67 L/L_0. The muscles were allowed to equilibrate for $\frac{1}{2}$ hr in seawater (pH 6.5) at 0–4°C. For each length, some muscles were fixed "at rest" and others were fixed "during activity". The "during activity" muscles were stimulated with square-wave pulses of 100V, 5 msec duration at 5 c/sec from a Grass S44 stimulator to obtain a sustained isometric tetanic contraction, and fixed immediately. The "at rest" muscles were fixed without stimulation. All muscles were fixed in 2.5% glutaraldehyde in 0.1M cacodylate buffer (pH 6.5), 0.8M sucrose and 5% DMSO for 1 hr at 0–4°C. The fixed VPRM were then suspended in 25% KOH (w/v) for 16 hr, utilizing the method of Cooke and Fay (1972). Single fibers were obtained by exchanging the 25% KOH solution with fresh KOH solution, and subsequent gentle agitation of the suspension. Length dimensions of intact single fibers were determined with the use of Nikon SUKE phase-contrast microscope equipped with an ocular micrometer. Photographic records were obtained utilizing a Nikon SUKE phase-contrast microscope equipped with a Nikon Semiautomatic Microflex EFM camera attachment. Fold angle, segment length, fiber band width and diameter measurement of single fibers (see Fig. 2, Abercrombie *et al.*, 1984 for a diagram which shows these parameters) were determined from analysis of photographic records (negatives) utilizing a Dietzgen microfilm reader.

RESULTS

Scanning electron microscopy (SEM)

While not allowing one to look at the entire length of a particular fiber, the use of SEM has allowed a more detailed look at the configurations of individual cells. In particular the technique allows one to see three dimensional aspects of the surface of fibers and how the fibers fit together. Figures 1–5 are scanning electron micrographs at the same magnification to illustrate the configurations of the fibers at different relative lengths. Figure 1 is a scanning electron micrograph of a muscle fixed at 0.5 L/L_0 illustrating the extreme folded configuration at this length. Both teased fibers and the underlying fibers show the same configuration. In this micrograph, it is possible to see that the folding is in three dimensions, giving the fibers a spiral or helical appearance. Figure 2 is a muscle fixed at 1.0 L_0, again showing the folded or spiral configuration of many teased fibers with the folds or turns in register. The underlying tissue also shows the same configuration as the teased portion. Figure 3 is representative of muscles fixed at 1.3 L_0, showing the near absence of folding at this length and at all greater lengths (see also Fig. 5, Abercrombie *et al.*, 1984). Figures 4 and 5 show muscles fixed at 1.5 and 2.1 L/L_0 respectively, illustrating the absence of any folding except in fibers which were detached at one end during stretch. Grouping of many fibers into bundles can be seen, and torn or ruptured fibers are sometimes seen at 2.1 L_0 due to the extreme stretch.

KOH-isolated cells

Although the fine structural preservation of KOH-isolated cells is probably inferior to that seen in the glycerinated-fixed-teased muscle used for SEM, these cells provide much more useful information. It is only with this technique that two distinct categories of cells could be recognized. Furthermore, it can be shown that the large diameter fibers and the smaller diameter fibers behave differently. The large fibers are the only ones which fold, and it can be seen that the degree of folding is related to the relative length of the muscle from which they were isolated. Figure 6 shows that folding occurs only at lengths below 1.3 L/L_0. Figure 7 shows that small fibers remain relatively straight regardless of the muscle length. Since Figs 6 and 7 are printed at the same magnification, the size difference of the two fiber types is evident.

The complete isolation of the cells by KOH treat-

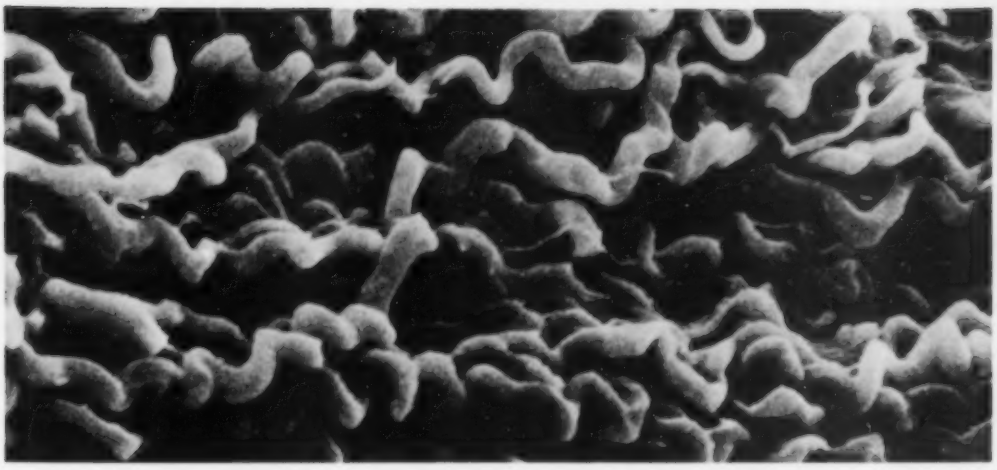

Fig. 1.

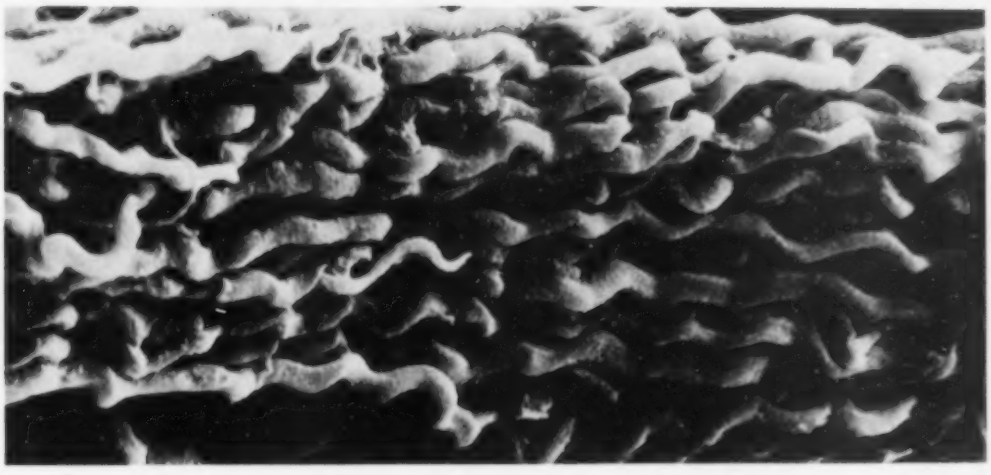

Fig. 2.

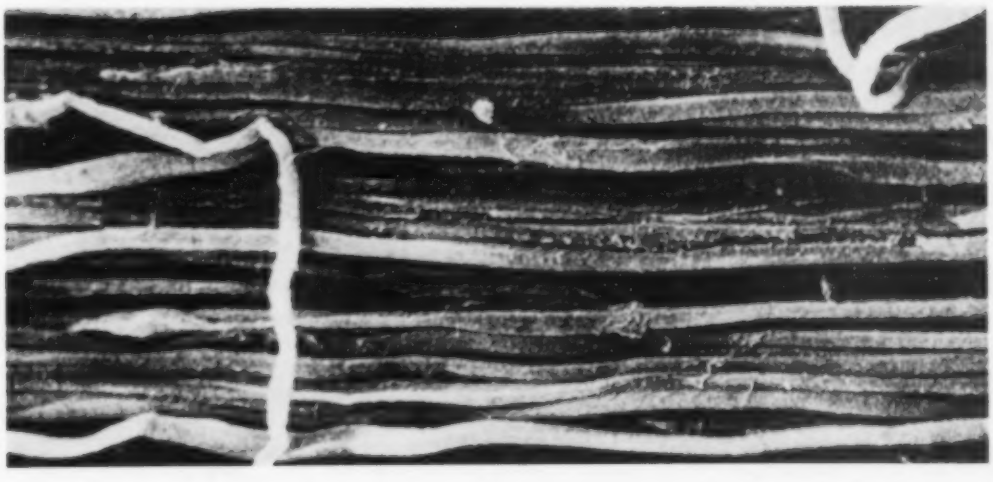

Fig. 3.

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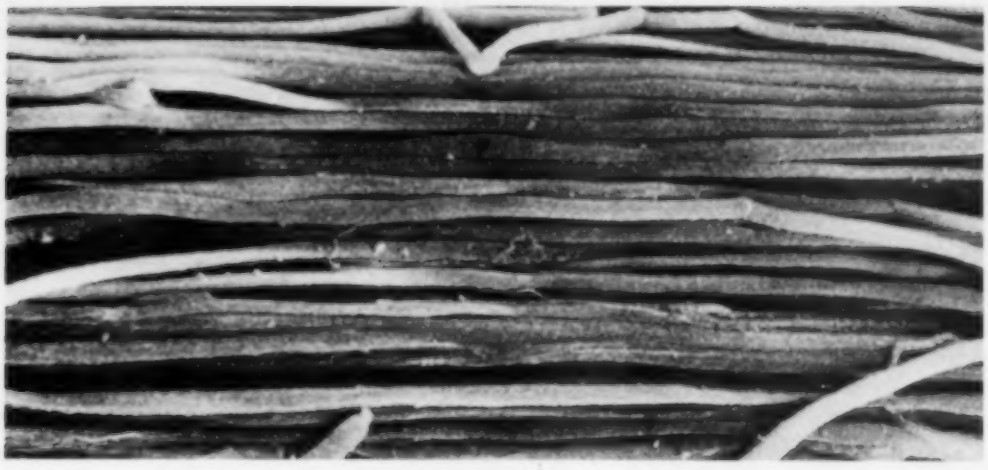

Fig. 4.

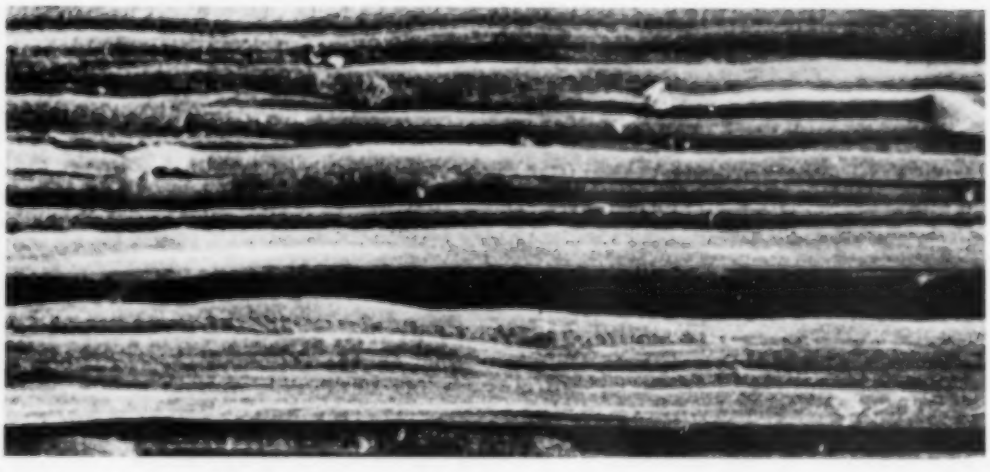

Fig. 5.

Figs 1–5. Scanning electron micrographs of muscles glycerinated at different relative muscle lengths (L/L_0) and teased gently to reveal the fibers. In the process of teasing, some superficial fibers are torn loose and are not representative, but underlying fibers retain their original configurations. Figs 1–5 show muscles at 0.5, 1.0, 1.3, 1.5 and 2.1 L/L_0, respectively. Note the helical configuration of some fibers in muscles at 0.5 and 1.0 L/L_0, the very slight folding at 1.3 and the absence of folding at 1.5 and 2.1 L/L_0. × 1000.

ment means that a variety of measurements may be made on individual cells. Table 1 shows the relationships between the relative length (L/L_0) of the muscle, the overall fiber length, fiber band width, fold angle, segment length and diameter of the *large* fiber as defined in the preceding paper (Abercrombie *et al.*, 1984). Note that the overall cell length is the length if the cell were unfolded, or the sum of the segment lengths. However, fiber band width is measured along the muscle axis and corresponds to the birefringent band width of the whole muscle, as described in the preceding paper (Abercrombie *et al.*, 1984). For the small fiber. Table 2 shows only the relationships between relative length (L/L_0) of the muscle, fiber length and fiber diameter, since the small cell does not fold. Beyond 1.5 L/L_0, the small cells become damaged and fragmented, so data for greater lengths were not included. It can be easily seen that for most values of relative muscle length, the diameter of the small fiber is significantly less ($P < 0.05$) than the diameter of the large fiber, but the lengths of the two types are very similar.

Figure 8 shows the relationship between relative muscle length (L/L_0) and fiber diameter of both large and small fibers "at rest" and "during activity". An increase in diameter may be used as a criterion for active contraction whereas an unchanged diameter indicates a lack of active contraction. The portion of the graph showing the large cell illustrates much the same phenomenon seen in the living cells in the whole muscle (Abercrombie *et al.*, 1984). That is, the KOH-isolated large cell shows progressively less active contraction at lengths below 1.3 L/L_0. The lower portion of the graph shows that the small fiber exhibits significant active contraction at 0.5 and 1.0 L/L_0, but at 1-5 L/L_0 (and presumably at greater lengths) there is no significant active contraction by small cells. Thus, the small fibers should be able to

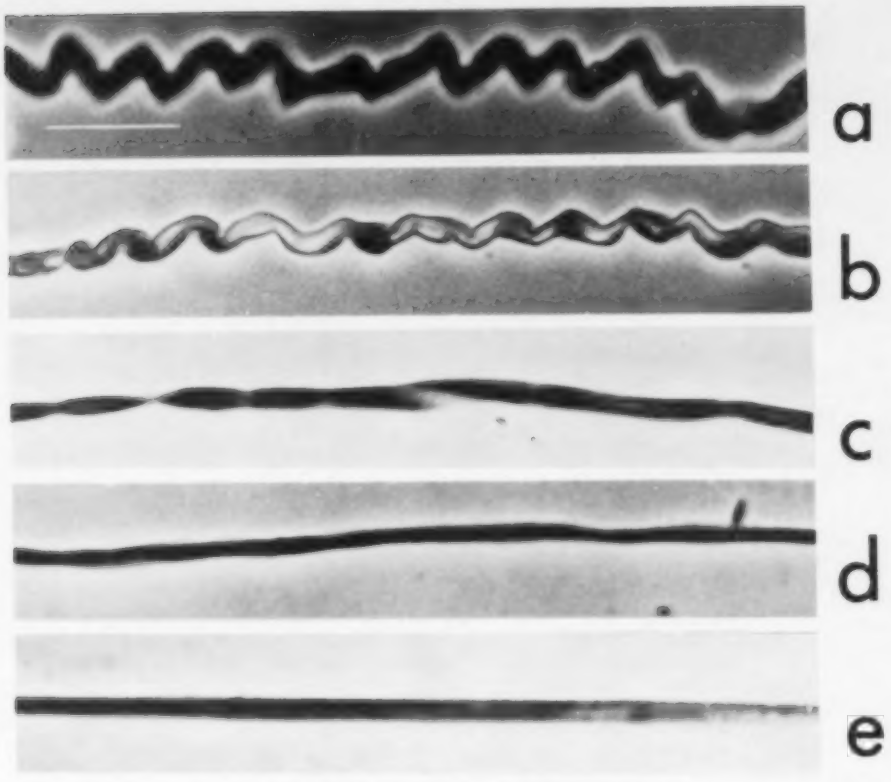

Fig. 6. Phase-contrast micrographs of large fibers isolated from muscles fixed at different relative muscle lengths (L/L_0) and treated with KOH. Figs 6a–e show fibers isolated from muscles fixed at 0.5, 1.0, 1.3, 1.5 and 1.67 L/L_0, respectively. Note the folding at 0.5 and 1.0 L/L_0, the slight folding at 1.3 L/L_0 and the absence of folding at greater lengths. Compare with the fibers seen in Figs 1–5. Dimension bar = 20 μm. ×1000.

exert the majority of their force in the length domain of first tension peak at 1.0 L/L_0, while the large fibers should exert the majority of their force at lengths greater than 1.3 L/L_0, in the length domain of the second peak at 1.67 L/L_0.

Another indication of whether the large fiber is active at the shorter lengths is to plot the calculated length of the large fiber (the length of the folded fiber if unfolded) against relative muscle length to see whether the large fiber actively shortens in the length range where it is folded. Figure 9 shows that the large fibers from *active* muscles are shorter at all muscle lengths except 0.5 L/L_0. These results are not in complete agreement with those on diameter measurements (see Fig. 8) since one would expect to see progressively less active shortening of the large cells

Fig. 7. Phase contrast micrographs of small fibers isolated from muscles fixed at different relative muscle lengths (L/L_0) and treated with KOH. Figs 7 a–c show fibers from muscles fixed at 0.5, 1.0 and 1.5 L/L_0, respectively. Note the absence of folding at all muscle lengths and the consistently smaller diameter (printed at the same magnification as Fig. 6). ×1000.

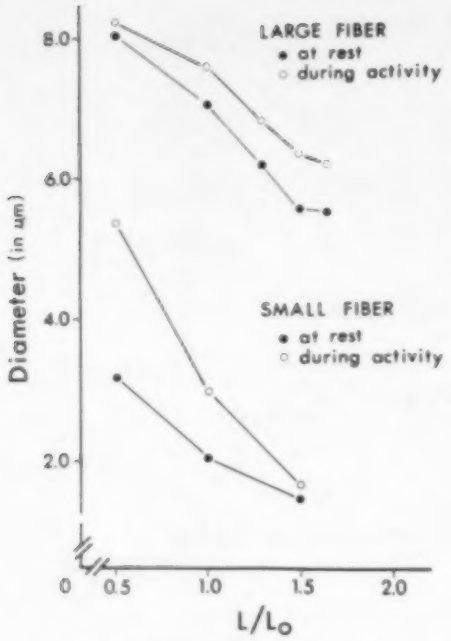

Fig. 8. A graph showing the relationship between the relative length of the muscle (L/L_0) and the diameter (in μm) of both the large fiber at rest (●) and during activity (○) and the small fiber at rest (●) and during activity (○) from KOH-isolated cells. Note the large fiber shows the greatest diameter change in the region of the second peak of the length–tension curve (1.67 L/L_0) and the small fiber shows the greatest change in the region of the first peak of the length–tension curve (1.0 L/L_0) and at shorter lengths.

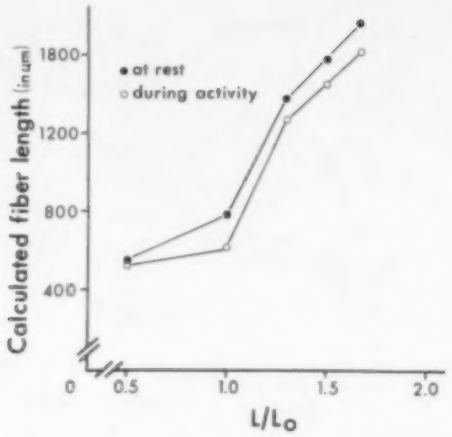

Fig. 9. A graph showing the relationship between the relative muscle (L/L_0) and the calculated length (if unfolded) of the large fiber at rest (●) and during activity (○) from fibers isolated by KOH treatment. The region where the fiber length tends to level out corresponds to the region where fiber folding occurs.

below 1.3 L/L_0. A portion of the "active" shortening in the length range where folding occurs (0.5–1.3 L/L_0) may be attributed to the increased folding or coiling of the large cells when the whole muscle contracts. Since the turns do not necessarily occur in the XY plane, some segments would be at angles to the plane of the slide leading to an underestimation of segment length and a corresponding over-estimation of active shortening. It is also interesting that the calculated large cell length is not linearly related to muscle length except in the region where the large cells are straight. Below 1.3 L/L_0 there is progressively less decrease in calculated large cell length with respect to decrease in muscle length, even in resting muscle. This is another indication that the large cell is out of its working range at the shorter muscle lengths.

DISCUSSION

The present paper was an extension of the study with living muscles (Abercrombie *et al.*, 1984) in an attempt to gain additional information on individual cell configurations.

SEM corroborated the relationship between folding of the large fiber and the relative muscle length, with folding occurring only at lengths below 1.3 L/L_0 in fixed cells as in living cells (see Fig. 5, Abercrombie *et al.*, 1984). However, the SEM micrographs of teased muscles at 0.5 and 1.0 L/L_0 provided the additional information that the "folds" of cells may be turns in a helix. This leads to some cautions in interpretation of measurements from folded cells, especially the measurement of segment lengths, since not all segments will be at the same angle to the optical axis. However, for simplicity of most analyses, the turns were assumed to be simple folds. SEM did not reveal the presence of a smaller fiber type,

Table 1. Large fiber measurements from muscles at different relative muscle lengths (L/L_0)

L/Lo	Length (Overall in μm)	F.B.W. (μm)	Fold Angle θ	Segment Length (μm)	Diameter (μm)
0.5	470±90	21.6±4.7	44.2±6.3	12.6±2.5	8.1±0.6
1.0	780±95	28.5±3.7	25.0±4.0	14.6±2.3	7.1±1.0
1.3	1380±320	N/A*	0°	N/A	6.2±0.9
1.5	1580±170	N/A	0°	N/A	5.6±0.8
1.67	1770±180	N/A	0°	N/A	5.5±0.9

*Not applicable in unfolded fibers. $N = 50$ for length measurements, $N = 25$ for all other parameters. All measures expressed as ± one standard error of the mean.

Table 2. Small fiber measurements from muscles at different relative muscle length (L/L_0)

L/Lo	Length (μm)	Diameter (μm)
0.5	380±70	3.2±0.8
1.0	710±140	2.0±0.5
1.5	1070±130	1.5±0.2

$N = 50$ for length measurements, $N = 25$ for diameter measurements. All measures expressed as ± one standard error of the mean.

perhaps because the smaller fibers may lie deeper within the muscle, and teasing only exposed superficial layers. The exact disposition of small fibers needs further study with transmission electron microscopy (TEM).

The KOH cell isolation procedure (Cooke and Fay, 1972) revealed two distinct populations of fibers, based on diameter measurements (see Figs 6 and 7, Tables 1 and 2). This confirmed observations by Ernst (1970) on *Phascolopsis* and by Reger (1964) on *Sipunculus* that proboscis retractors of sipunculids contained two fiber types.

Since all cell measurements in the present paper are related to the length (L/L_0) of the muscle from which they were obtained, and it is essential to know whether the cells from muscles fixed at different L/L_0 were fixed in the length domain of the first peak or the second peak in the length-tension relationship, the reader is urged to consult Fig. 4a in the preceding paper (Abercrombie *et al.*, 1984) which shows the length–tension relationship of muscles which were used both in the study of living whole muscles and in the present study. By graphing the parameters measured from the KOH study against relative muscle length (L/L_0) several conclusions can be arrived at as to the mechanism of contraction in the proboscis retractor muscle. Using diameter increase in cells from stimulated muscles as an indicator of active contraction by the cell, the influence the cell contributes at that particular point on the length–tension relationship can be determined. The large fiber's influence was determined to be mainly at the second peak ($1.67\ L/L_0$) of the length–tension relationship. The data on the large fiber analyzed from the KOH study are in agreement with those from the previous studies of the living intact muscle. Specifically, active contraction occurred only at $1.0\ L/L_0$ and greater lengths according to the KOH data, and at $1.15\ L/L_0$ and greater lengths according to the data from living muscles (Abercrombie *et al.*, 1984). However, the small fiber was not visible in the living studies, and the KOH study provided the sole data about its behavior at different muscle lengths. The influence of the small fiber in tension production should be in the region of $0.5–1.5\ L/L_0$ and not at lengths beyond since there was no diameter increase in active small fibers at $1.5\ L/L_0$, and irreversible damage to the small fiber occurs at lengths beyond $1.5\ L_0$. Irreversible damage has also been shown by the Matsumoto and Abbott (1968) length–tension study on hysteresis. They found that after stretching in the length region where passive tension rises (about $1.5\ L/L_0$), active tension measurements were markedly attenuated when the length of the muscle was changed in the reverse direction (i.e. shortened in increments).

Therefore, in the length–tension relationship, the large cells are mostly responsible for the second peak at $1.67\ L/L_0$ and small fibers are unable to participate in force production except in the initial portion of the ascending limb (between 1.3 and $1.5\ L/L_0$) (see Fig. 4a, Abercrombie *et al.*, 1984). The diameter changes in the small fibers indicate they are active in the region of $0.5–1.0\ L/L_0$ with decreasing activity between 1.0 and $1.5\ L/L_0$. The small cells should have their highest force production between 0.5 and $1.0\ L/L_0$, meaning that they provide a large part of the force for the first peak at $1.0\ L/L_0$. But what about the role of the large fibers in the first peak? The large fibers were shown to have some degree of activity at $1.0\ L/L_0$ (see Fig. 8). However, there are two reasons for concluding that their role in force production under the first peak must be minimal. The fold angles at these lengths decrease the amount of force transmitted in the longitudinal axis since force in the folding fibers is a function of cosine θ (see Fig. 2, Abercrombie *et al.*, 1984). As the fold angle increases, the contribution to longitudinal force decreases markedly. If the large fibers are responsible for the second peak at $1.67\ L/L_0$, this would mean that this peak represents *their* L_0. Therefore, at $1.0\ L/L_0$ for the whole muscle, the large fibers would be at 60% of their L_0 (the length at which they produce their maximum force). For vertebrate cross-striated fibers, a length of 60% L_0 results in a force production near zero (Gordon *et al.*, 1966). The pedal retractor muscles of *Mytilus edulis* have a greater range of lengths for force development (Ishii and Takahashi, 1982), not dropping to zero force until 35% L_0, and in vertebrate smooth muscles there are several examples where force may be exerted below 50% L_0 (Åberg and Axelsson, 1965; Cooke and Fay, 1972; Driska *et al.*, 1978; Meiss *et al.*, 1974; Uvelis and Gabella, 1980). However, these muscles generally have rather broad peaks, while the peaks obtained from the VPRM are sharper. Extrapolation along the slope of the second peak indicates that the larger fibers should be producing almost no force at $1.0\ L/L_0$ where the first peak occurs. Therefore, we conclude that the individual peaks are essentially produced by different types of fibers, with the first peak produced by small fibers and the second peak produced by large fibers, with contributions by both fibers in the area between peaks.

It was suggested in the preceding paper (Abercrombie *et al.*, 1984) that the folding fibers folded passively, with the force for folding provided by a population of straight cells. The present investigation has demonstrated that a population of smaller diameter fiber exists, that these fibers are active in the length range where folding occurs ($0.5–1.3\ L/L_0$), and that these fibers remain straight in the length range where folding occurs. We cannot rule out some force production by the large fibers in the length range where they fold, but lacking a suitable mechanism whereby this force could be translated into significant longitudinal force, and having a considerable force production by the small cells in this length range, we

conclude that the small fibers provide the force which causes the large fibers to fold.

It is somewhat paradoxical that the small fibers produce the largest peak and the large fibers produce the smaller peak in the bimodal length–tension curve (see Fig. 4a, Abercrombie *et al.*, 1984). This means that the small cells are more numerous and/or are capable of greater force production per fiber. The small fibers are known to contain large paramyosin filaments, while the large fibers do not (Ernst, 1970), so it is quite possible they have different force production. A study of cross-sectioned muscle would help resolve the question of relative numbers as well as providing information on the disposition of the two fiber types. It would be very helpful if a single fiber mechanical study could be done, as was done by Ishii and Takahashi (1982) with pedal retractor muscle of *Mytilus edulis*. This would allow comparisons of force production in the two fiber types, as well as providing additional information on the length ranges of tension development in the small and large fibers.

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TISSUE PRODUCING THE SERUM FACTOR STIMULATING THE RELEASE OF CATHEPSIN D FROM LYSOSOMES *IN VITRO*

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Abstract—1. Pancreatectomy as well as thyroparathyroidectomy resulted in the quick disappearance of a serum factor (stimulating cathepsin D release from lysosomes *in vitro*) from the rat or mouse blood. Extirpation of other organs such as duodenum, stomach, spleen, kidney, submaxillary gland, testis, adrenal gland or hypophysis, showed no effect on the serum factor level.

2. Glucagon (but not insulin or thyroxine) given to the pancreatectomized animals restored the serum factor level in a dose-dependent manner.

3. The serum factor-like activity was detected only in the parathyroids (but not thyroid), and the release of activity from parathyroid-slices was stimulated by glucagon, suggesting that the parathyroid may produce and/or secrete the serum factor under the influence of glucagon.

INTRODUCTION

In the course of studying the mechanisms of liver regeneration, we found that the pleiotypic changes after partial hepatectomy are markedly inhibited and/or retarded by intraperitoneal injections of some protease-inhibitors such as leupeptin and pepstatin (Miyamoto *et al.*, 1973), suggesting the possible involvement of some endogenous protease(s) in the initiation of hepatocyte proliferation *in vivo*.

Looking for a factor(s), which may stimulate the release of cathepsin(s) from isolated lysosomes *in vitro*, we eventually found a serum factor (heat-stable, low molecular weight) stimulating cathepsin D-release from lysosomes isolated from rat livers (Miyamoto and Terayama, 1975). Later, we have found that the heated serum extract or partially purified preparations therefrom can stimulate the release of cathepsin L as well, but not cathepsin B or acid phosphatase (Miyamoto and Terayama, in preparation).

Quite interestingly, the serum factor level in the blood was enhanced (> 2-fold) within several hours after 70% hepatectomy with concomitant elevation of blood cathepsin D. Moreover, an intraperitoneal injection of a serum factor preparation (from the bovine serum) into mice gave rise to the immediate rise of cathepsin D level in the blood, which was followed by the stimulated DNA synthesis and mitosis in the liver in accordance with the initiation of liver cell proliferation by an intraperitoneal injection of cathepsin D and L (but not cathepsin B) (Miyamoto and Terayama, submitted) much like the effect of papain (Kambara and Nohara, 1966; Yamamoto *et al.*, 1973) reported earlier.

The present study is mainly concerned with the elucidation of a tissue or organ producing the serum factor stimulating the release of cathepsin D from isolated lysosomes.

MATERIALS AND METHODS

Preparation of lysosomal suspension

Lysosomes were isolated from the liver of male, Wistar rats weighing 120–150 g. The liver homogenate in 8 vol 20 mM Tris–HCl buffer (pH 7.2)–0.25 M sucrose was subjected to differential centrifugation (750 *g* for 10 min, 3300 *g* for 10 min, and 15,000 *g* for 20 min) to collect the final pellet ("light-mitochondrial"), which was then quickly rinsed with the 0.25 M sucrose–1 mM EDTA (pH 7.2) solution, and resuspended in the same solution (20 ml/10 g liver).

Assay of serum factor

The serum factor activity was assayed as described earlier (Miyamoto and Terayama, 1975) based on the stimulatory effect on cathepsin D-release from lysosomes. The residual cathepsin D activity (activity remained in lysosomes after 1 hr of incubation at 37°C) was measured instead of measuring cathepsin D activity released into the medium because of a partial inactivation of the released enzyme during incubation. The reaction mixture consisting of 2.0 ml of the lysosomal suspension and 0.6 ml of a test solution or 0.9% NaCl (the control) was centrifuged (15,000 *g*, 20 min) after 1 hr incubation, and the pellet was solubilized with 2.0 ml of 0.2% Triton X-100 in 0.25 M sucrose at 0°C for 1 hr, followed by centrifugation (15,000 *g*; 20 min) to separate the supernatant (residual activity). Another aliquot of the lysosomal suspension was treated with Triton without incubation and centrifuged to separate the supernatant (total activity). Cathepsin D activities in the supernatants were assayed according to the method of Anson (1938) using a 0.1 ml aliquot of each supernatant and 1.9 ml of acid denatured hemoglobin in the 0.18 M acetate buffer, pH 3.2. After incubation at 37°C for 1 hr, the reaction was stopped by adding cold 10% trichloroacetic acid and the supernatant was subjected to the Lowry colorimetry at 750 nm (Lowry *et al.*, 1951).

The percent release of cathepsin D was defined as 100 × (total activity − residual activity)/total activity. The percent stimulation of cathepsin D-release by a test material was defined as 100 × (% release in the presence of test material −% release in the control)/% release in the control. One unit of the serum factor activity corresponds to 10% stimulation. It has been confirmed that a linear correlation is found up to 100% stimulation.

Preparation of test solutions from various organs or tissues

Organs or tissues from rats (male, Wistar), mice (male, ddN) or cows (obtained from a local butchery) were homogenized with an equal volume of water, and the homogenates were successively centrifuged; 15,000 *g* for 20 min and then 105,000 *g* for 90 min. The last supernatant was heated at

100°C for several minutes and then spun down (15,000 *g* for 20 min) to separate the supernatant (heated tissue-extract). The heated tissue-extract was evaporated to dryness under reduced pressure and the residue was suspended in an adequate amount of water. The suspension was twice washed with an equal volume of ether, and the aqueous phase was evaporated to dryness. The residue was dissolved in an adequate amount of water, and was assayed for serum factor-like activity.

Release of serum factor-like activity from parathyroid slices

The bovine parathyroids from several cows were cut into slices with 0.5 mm-thickness within 3 hr after killing (the organs were kept over ice after excision). Slices of about 4 g (wet tissue) (randomly mixed among animals) were quickly rinsed with the Ca^{2+}-free Hank's solution and then suspended in 20 ml of the Ca^{2+}-free Hank's solution fortified with Ca^{2+} or glucagon in various concentrations. After incubation at 37°C for scheduled intervals of time, a 0.5-ml aliquot of the incubation medium was withdrawn, heated at 100°C for several min, and then centrifuged (15,000 *g*, 20 min) to separate the supernatant (released activity). At the end of incubation, the remaining tissue-slices were picked up, homogenized with water (10% homogenate), and then successively centrifuged (15,000 *g*, 20 min and 105,000 *g*, 90 min). The supernatant was heated and then centrifuged as described earlier to separate the supernatant (residual activity). Percent release of serum factor-like activity was defined as 100× (released activity)/(released activity + residual activity at the end of incubation).

Thin-layer chromatography

Thin-layer chromatography was carried out with a partially purified preparation of serum factor (from the bovine serum) and a heated parathyroid-extract (bovine) after ether washing using a thin layered (0.1 mm thickness) cellulose on an aluminium plate (F 254, A. Merck, Darmstadt, Germany) and a solvent system of *n*-butanol : acetic acid : water (12 : 3 : 5 v/v) at room temperature. The plate was then sectioned every 0.5 cm distance and each section was 3 times extracted with water (each 3 ml). The extracts were combined, evaporated to dryness and assayed for the serum factor activity.

Hormones

Glucagon (crystalline, bovine pancreas, No. G-4250; Sigma Chem. Co., St. Louis, USA), insulin (crystalline, bovine pancreas, No. 15500; Sigma Chem. Co., St. Louis, USA) and L-thyroxine–Na (Wako Pure Chem. Ind., Ltd., Tokyo, Japan) were purchased from local dealers.

RESULTS

Effect of extirpation of various organs on the level of serum factor in rat or mouse blood

Sera were prepared from the bloods of rats or mice, which had received surgical removal of a certain organ, and were assayed for the serum factor activity. As summarized in Table 1, pancreatectomy as well as thyroparathyroidectomy appeared to give rise to the remarkable drop of serum factor level within 30 hr after operation in contrast to the significant increase in the serum factor activity in partially hepatectomized rats.

Table 1. The level of serum factor in the peripheral blood after extirpation of various organs

Organ extirpated	Time after extirpation (h)	Serum factor level (units/ml serum)
None (R)		57 ± 8 (41)
None (M)		51 ± 4 (2)
Thyroid and parathyroid (R)	21	13 ± 0 (2)
	31	3 ± 3 (4)
Pancreas (R)	43	6 ± 2 (2)
Pancreas (M)	33	3 ± 2 (4)
Spleen (R)	23	52 ± 7 (6)
Kidney (R)	31	54 ± 6 (3)
Duodenum (R)	23	50 ± 11 (5)
Stomach (R)	21	61 ± 4 (3)
Submaxillary gland (R)	31	64 ± 8 (3)
Testis (R)	31	42 ± 9 (3)
Adrenal gland (R)	48	59 ± 1 (4)
Hypophysis (R)	72	52 ± 1 (2)
Liver (70%) (R)	4	67 ± 7 (2)
	6	70 ± 7 (2)
	10	49 ± 1 (2)

Mice (M) or rats (R) received the surgical removal of a certain organ(s). Sera were prepared from the peripheral blood at intervals after surgery, and assayed for the serum factor activity. Mean ± SD. (Number of animals.)

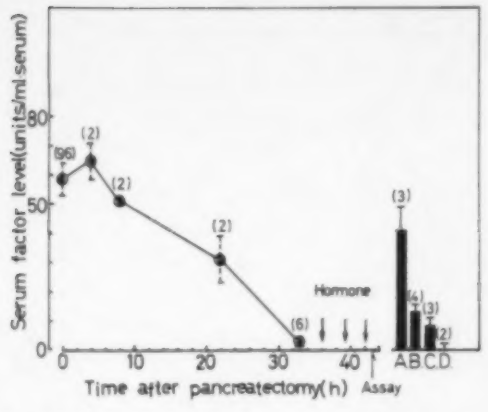


Fig. 1. Effects of pancreatectomy and hormonal injections into pancreatectomized mice upon the serum factor level in the blood. The serum was prepared from each mouse at various intervals of time after extirpation of total pancreatic tissue (Scow, 1957) and assayed for the serum factor level as described in the text. Starting from 36 hr after pancreatectomy, the mice received 3 successive intravenous injections of glucagon (A) (each 0.05 mg/head), insulin (B) (each 0.1 mg/head), thyroxine (C) (each 0.1 mg/head) or saline (D) with a 3-hr interval, and the serum was prepared 1 hr after the last injection, followed by assaying the serum factor. Each point is the mean of several mice (number in parentheses) with SD.

The time–course change of serum factor level in pancreatectomized mice is shown in Fig. 1.

Effects of some hormones on the serum factor level in pancreatectomized mice

Starting from 36 hr after pancreatectomy, the mice received three successive intravenous injections of insulin (each 0.1 mg/head), glucagon (each 0.05 mg/head), L-thyroxine (each 0.1 mg/head), or the saline (vehicle; 0.5 ml/head) with an interval of 3 hr. The serum factor level in each mouse was assayed 1 hr after the last injection. As also shown in Fig. 1, the serum factor level once dropped in pancreatectomized mice was markedly elevated by glucagon injections, whereas the level remained low in the mice which received insulin, thyroxine or saline.

The recovery of serum factor level appeared to be dependent on the dose of glucagon as shown in Fig. 2, indicating that 0.05 mg of glucagon (sum in 3 injections) was large enough to restore the serum factor up to the normal level. It should be noted that glucagon injected into normal mice did not change the normal level of serum factor or the effect of injections of insulin plus glucagon was much like the effect of injections of glucagon alone (data not shown).

Serum factor-like activity in the heated tissue-extracts from various organs or tissues

The heated tissue-extracts were prepared from various organs or tissues (rat, mouse or bovine) as described earlier and assayed for the serum factor-like activity. As summarized in Table 2, the heated tissue-extracts from various organs or tissues before ether washing showed more or less serum factor-like activities. However, the appreciable activities were detected only in the heated parathyroid-extract after ether washing much like the serum.

Figure 3 shows the chromatograms of the heated parathyroid-extract and the partially purified serum factor preparation, indicating 3 major activity bands in each sample.

Effects of glucagon and Ca^{2+} on the release of serum factor-like activity from parathyroid slices

As shown in Fig. 4(a), considerable portions of serum factor-like activity seem to be quickly released from parathyroid slices into the medium (Ca^{2+}-free Hank's solution) during incubation, and the release seems to be significantly stimulated by glucagon in a concentration of about 1 μg/ml (or 2.7×10^{-7} M). The release of serum factor-like activity from parathyroid slices appeared to be dependent on the concentration of glucagon added to the incubation medium within a concentration range of 2.7×10^{-10} to 2.7×10^{-8} M as shown in Fig. 4(b).

The release of serum factor-like activity from parathyroid slices was found to be stimulated also by Ca^{2+} added to the Ca^{2+}-free Hank's solution as shown in Fig. 5, indicating a half maximal stimulation at 0.05–0.10 mM Ca^{2+}. It should be noted that the addition of Ca^{2+} to the medium together with glucagon does not give any additive effects, and the glucagon effect becomes less marked in the presence of Ca^{2+} (data not shown), suggesting that the glucagon effect may be mediated by the intracellular Ca^{2+}.

DISCUSSION

In this paper we showed that a serum factor having the activity to stimulate cathepsin D release from lysosomes (rat liver) *in vitro* may be produced and/or secreted in the parathyroids under the influence of pancreatic glucagon mainly based on the following findings; (1) thyroparathyroidectomy as well as pancreatectomy gives rise to the quick disapperance of serum factor in the blood (Table 1 and Fig. 1), (2)

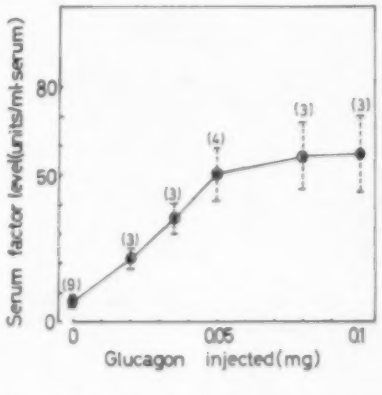


Fig. 2. Glucagon dose-dependency in the recovery of serum factor level in pancreatectomized mice. Starting from 36 hr after pancreatectomy, each mouse received 3 intravenous injections of glucagon with a 3-hr interval. One hour after the last injection, the level of serum factor (ordinate) was assayed. Abscissa indicates the total amount of glucagon administered. Each point is the mean of several mice (number in parenthesis) with SD.

Table 2. Serum factor-like activities of the heated tissue-extracts from various organs or tissues

Organs or tissues	Serum factor-like activity (units/g tissue) Before ether washing	After ether washing
Duodenum (R)	135 ± 9 (21)	0 (8)
Pancreas (R)	132 ± 18 (12)	5 (4)
Pancreas (M)	141 ± 5 (18)	N.D.
Small intestine (R)	49 ± 10 (12)	0 (2)
Spleen (R)	52 ± 4 (12)	2 (2)
Stomach, whole (R)	50 ± 7 (6)	9 (3)
Stomach, pyloric (R)	63 ± 6 (3)	N.D.
Adrenal gland (R)	38 ± 10 (3)	2 (8)
Liver (R)	53 ± 2 (5)	0 (1)
Adipose tissue (R)	61 ± 8 (8)	N.D.
Brain (R)	54 ± 3 (8)	N.D.
Kidney (R)	59 ± 3 (16)	0 (6)
Parathyroid (B)	46 ± 8 (56)	34 ± 8 (72)
Thyroid (B)	48 ± 3 (66)	4 ± 3 (24)
Serum (R)	57 ± 8 (123)	55 (2)

The heated tissue-extracts were prepared from the organs or tissues of animals and assayed for the serum factor-like activity before and after ether-washing as described in Materials and Methods. Mean ± SD. (Number of animals). R, rat; M, mouse; B, bovine. N.D., not determined.

administration of glucagon restores the serum factor level in the pancreatectomized animals (Fig. 2), (3) the serum factor-like activity is detected only in the parathyroids (bovine) (Table 2), and (4) the release of serum factor-like activity from parathyroid slices is stimulated by glucagon in a physiological concentration as well as Ca^{2+} (Figs 4 and 5).

In our laboratory we have observed that an extensively purified serum-factor preparation (from bovine serum) can stimulate the release of cathepsin D-like activity from erythrocytes or ghosts (rats) much like lysosomes, and the name of "calciferin" was given to the active principle acting on the erythrocytes or ghosts (Takehara and Terayama, submitted because the active principle acting on the erythrocytes or ghosts (Takehara and Terayama, submitted) because plasma membranes.

Although the identity of active principle(s) acting on lysosomes and that on erythrocytes or ghosts *in vitro* as well as stimulating liver cell proliferation *in vivo* (as stated earlier) remains to be clarified by further investigations, it may be worthwhile to note that a quite satisfactory correlation has so far been obtained with various serum factor preparations of different specific activities with respect to these *in vitro* and *in vivo* activities.

The finding that the secretion of serum factor-like activity from the parathyroids is dependent on pancreatic glucagon as well as Ca^{2+} seems to be particularly interesting although the presence of glucagon receptor in parathyroids remains to be investigated. The level of glucagon in the blood is known to be enhanced immediately after partial hepatectomy

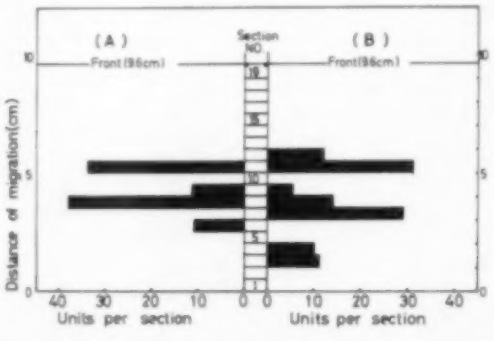
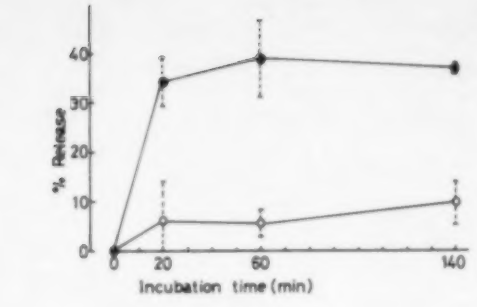
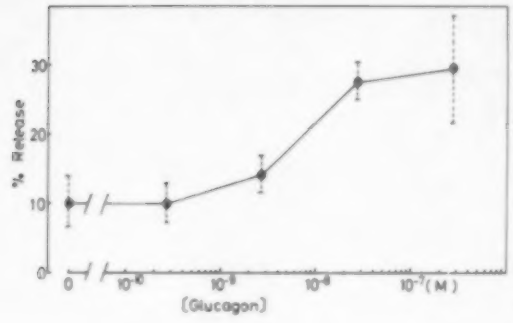


Fig. 3. Thin-layer chromatography of a partially purified preparation of serum factor (from the bovine serum) (A) and the heated parathyroid-extract (bovine) after ether-washing (B). The samples were developed on a thin layered cellulose plate (aluminium) (F-254, Merck) in *n*-butanol:acetic acid:water (12:3:5 v/v) until the front migrated a 9.6 cm distance. The plate was cut into sections every 0.5 cm, and each section was extracted with water, followed by assaying the serum factor activity in each extract as described in the text.



(a)



(b)

Fig. 4. (a) Time course of the release of serum factor-like activity from parathyroid slices in the presence and absence of glucagon (2.7×10^{-7} M). (b) Glucagon concentration-dependency of the release of serum factor-like activity from parathyroid slices as measured at 60 min of incubation. Each point is the mean ±SD of duplicate determinations.

(Leffert *et al.*, 1979). Glucagon, which stimulates adenylate cyclase in hepatocytes (Birnbaumer *et al.*, 1971), is also known to stimulate hepatocyte proliferation to some extent (Short *et al.*, 1972). Adenylate cyclase in hepatocytes is also known to be activated by some mitogenic proteases (Koji and Terayama, 1980)

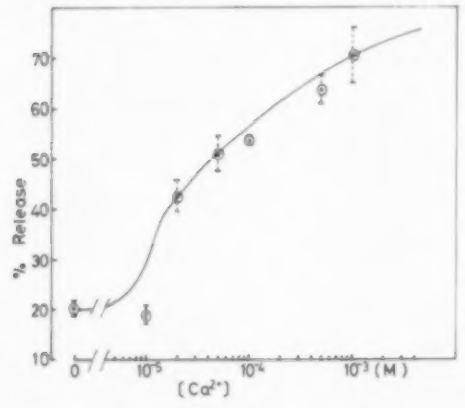


Fig. 5. Ca^{2+} concentration-dependency of the release of serum factor-like activity from parathyroid slices as measured at 60 min of incubation. Each point is the mean ±SD of duplicate determinations.

in accordance with the findings indicating the intimate involvement of cyclic AMP in the initiation of hepatocyte proliferation (Rixon and Whitfield, 1976). These earlier observations together with the present one seem to suggest that the pancreas as a main glucagon producer is a detector of the reduction of liver size in partially hepatectomized animals, and stimulates the hepatocyte proliferation via dual mechanisms; (1) the direct action of glucagon on hepatocytes, and (2) the indirect action of glucagon via the serum factor and cathepsin(s) in the circulation. The above preposition seems to be also in accord with the inhibition of liver regeneration in the pancreatectomized (Bucher and Swaffield, 1975; Bucher and Weir, 1976) or hyperglycemic (Tsuzuki, 1967) animals.

Although the chemical identification of serum factor remains to be investigated, it should be noted that our serum factor is not related to γ-glutamyl taurine, which has recently been claimed to be a new parathyroid hormone (Feuer *et al.*, 1979) because γ-glutamyl taurine did not show any effects on the release of cathepsin D from lysosomes or from erythrocyte ghosts, and behaved entirely differently in chromatography on ion-exchange resins (data will be reported in a separate paper).

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DISTRIBUTION OF NEUTRAL LIPIDS IN COELOMIC CONSTITUENTS DURING OOGENESIS OF *PERINEREIS CULTRIFERA* (ANNELIDA POLYCHAETA)

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(*Received* 4 *March* 1983)

Abstract—1. The oocyte growth of *Perinereis cultrifera* (Polychaeta) is accompanied by considerable variations in lipid composition of oocytes, coelomocytes and coelomic fluid.

2. Oocytes are rich in lipids (between 20 and 40% of their total dry weight). The rate of total lipids of coelomocytes increases very clearly during oogenesis.

3. Monoacylglycerols and diacylglycerols are present in large proportions only in coelomic fluid. There are many triacylglycerols in coelomocytes and oocytes, but the proportions vary considerably during the evolution of the oocyte: their rate increases in coelomocytes but decreases in oocytes. Deprived of brain neurohormone, triacylglycerols increase in coelomocytes, but there is no variation in oocytes.

4. The rate of free sterols increases in the same way in coelomocytes and coelomic fluid. Sterol esters begin in coelomocytes before constituting a high and constant level in neutral lipids of coelomic fluid. In the absence of the brain hormone, coelomocytes steryl esters of young *P. cultrifera* decrease in rate considerably, as during genital maturity in natural conditions.

5. The role of coelomocytes in the synthesis of substances, discharged into the coelomic fluid and involved in the development of oocytes is discussed.

INTRODUCTION

In Nereidae, oocytes are free in the coelomic cavity: they form their reserves of yolk and cortical alveolis by direct uptake from the coelomic fluid. A cerebral neurohormone inhibites oogenesis and the somatic transformations of the epitoke; the rate decreases at the time of genital submaturity of the worm and increases at the onset of reproduction (Durchon and Porchet, 1970, 1971).

In the coelomic fluid, except for oocytes, there are free cells called coelomocytes (Baskin, 1974). Eleocytes, the most important of these cells, are rich in glycogen and lipid storage (Romieu, 1923; Dhainaut, 1966). Oocytes of the polychaete annelids are able to incorporate micromolecular precursors, amino acids or monosaccharides from the coelomic fluid to yolk formation (Dhainaut, 1967, 1968; Porchet *et al.*, 1977; Dhainaut and Porchet, 1977a,b). However, immunological studies have revealed an antigenic identity between coelomocytes, coelomic fluid and oocytes. Dhainaut *et al.* (1980a) and Fischer (1979) showed the existence of a vitellin-like antigen in the coelomic fluid of maturing *Nereis virens* females. So an exogenous way of yolk formation in Nereidae is possible. Eleocytes have long been suspected of playing a role in the maturation of the gametes in polychaetes (Kükenthal, 1885; Dales, 1957 and 1964; Dhainaut, 1967; Schroeder, 1971; Eckelbarger, 1974), but no morphological demonstration of nutrient transfer (i.e. pinocytosis by the oocyte) has been recorded.

After Telfer (1954) numerous reports have shown that vitellogenins are precursors of the major yolk proteins of insects, they are produced by fat body cells and exported into the haemolymph from where they are taken up by the growing oocytes. In annelids, if the transfer of nutrients occurs between coelomocytes and oocytes, it is presumably in a low mol. wt form, for there is no evidence from election microscopy to suggest gross uptake by the oocytes during vitellogenesis (Dhainaut 1966, in *Nereis pelagica*; Schroeder 1971, in *Nereis grubei*; Eckelbarger 1974, in *Nicolea zostericola*).

In this paper we study neutral lipids, and their variations during oogenesis, in the three coelomic constituents of *P. cultrifera* in order to investigate in more detail the metabolic interrelationships between coelomocytes, coelomic fluid and oocytes.

MATERIALS AND METHODS

Animals. Worms from the Biological Center of Luc-sur-mer (Calvados, France) were kept individually in filtered sea water, at a mean temperature of 12°C. Females of *Perinereis cultrifera* Grübe were used at different stages of development. Measurement of 30 oocytes, obtained by coelomic punction of an animal brought out the genital state (Durchon, 1952). Males of *Nereis diversicolor* O. F. Müller and *Arenicola marina* L. were used for some experiments.

Isolation of coelomic constituents. The coelomic contents of animals in the same genital state, harvested by punction with a glass pipette, were centrifuged for 10 min at 10,000 *g*. The supernatant was the coelomic fluid. Coelomocytes were isolated from oocytes by four washes in sea water followed by centrifuging for 3 min at 1000 *g* (Porchet, 1974).

Lipid extraction and analysis. Tissues were homogenized and coelomic fluid, coelomocytes and oocytes were lyophilized. Total lipids were extracted by the procedure of Folch *et al.* (1957) with chloroform–methanol (2:1, v/v). After two additional extractions, combined extracts were washed with 9% NaCl. The low chloroform phase, which contained lipids, was evaporated and weighed.

Neutral lipids fractionation and quantification. Aliquots of the crude lipid extracts were separated by thin-layer chromatography on silicagel G (Merck) using a solvent system of petroleum ether–ethyl ether–acetic acid (80:20:1, v/v). α-Monopalmitin, dipalmitin, tripalmitin, linoleic acid, cholesterol and cholesteryl palmitate, were used as standards. The bands were visualized by I_2 vapor retention or under u.v. light after spraying the plate with rhodamine 6 G (Sigma Chemical Co.), TLC being partially covered with a sheet of aluminum foil so that only lateral lanes remained exposed. Silica gel containing the lipid bands under study was scraped off the plate. The lipids were recovered by elution with chloroform methanol (2:1, v/v) and the amounts of neutral lipids were determined by the spectrophotometric procedures of Amenta (1964). For quantification, monoacylglycerol and diacylglycerol were combined.

RESULTS

Variation in the content of total lipids during oogenesis of Perinereis cultrifera

As shown in Fig. 1, there is a high rate of total lipids during submaturity (from an oocyte size of 180 μm) which constitutes 30% of the coelomic content on a dry weight basis. Lipids are particularly present in oocytes and coelomocytes. There are fewer lipids in coelomic fluid. At the end of oogenesis there is a clear decrease in the level of total lipids in all the coelomic constituents. Caner (1981) has also shown in *P. cultrifera* a large fraction of lipids in oocytes, and a decrease in rate at maturity. The results of studies on the total lipid content in males of *P. cultrifera* and *Nereis diversicolor* have shown that coelomic fluid is always a large fraction of the total dry weight of the coelomic content. Moreover, if lipids predominate in figured elements (in coelomocytes and gametes, they constitute 16–18% on a dry weight basis) they are also numerous in coelomic fluid (11–12%). Lastly, the percent distribution of lipids is similar in the coelomic constituents of the males of the two species of annelids.

Neutral lipid content and its variation during oogenesis (Table 1 and Fig. 2)

In coelomocytes the monoacylglycerols and diacylglycerols content (MG + DG) is low and remains at an almost constant level during oogenesis (5 ± 4%). The triacylglycerols (TG) vary in quantity but they always constitute a considerable ratio to neutral lipids. From 30% of neutral lipids at the stage 100–120 μm their rate increases up to maturity (at stage 200–220 μm the rate of TG is more than twice as high as in young worms). The neutral lipids of coelomocytes contain 20% of free fatty acids (FFA) at 100–120 μm, their rate increases gradually up to stage 180–200 μm and then decreases. The free sterols (S) content is low in young females but high at stage 180–200 μm (their rate is six times as high as in young *Perinereis cultrifera*). The esterified sterols (ES) have a very high content in coelomocytes of young worms, but it decreases rapidly and makes up only 1% of neutral lipids at maturity.

In the coelomic fluid the MG + DG content is high but it decreases during oogenesis (it constitutes 29% of neutral lipids in young worms, but 13% at maturity). There are traces of TG at stages 100–120 μm (only 1% of neutral lipids), but they vary considerably in quantity during oogenesis to constitute 17% of neutral lipids at submaturity. When the oocytes are mature the content level of TG decreases, and it constitutes only 5–6% of the lipids studied. The rate of FFA decreases gradually; the FFA content ranges from 44.5% at stage 100–120 μm to 14% at stage 220–240 μm. The free sterols content is low in the coelomic fluid of young worms. However, it increases until the end of oogenesis; it is 10 times as high at stage 220–240 μm as at stage 100–120 μm. ES are always very numerous (22.5–37% of neutral lipids).

In the oocytes, MG + DG make up a large fraction of neutral lipids only at submaturity. There are numerous TG in the oocytes but they decrease during oogenesis (from 77% at 100–120 μm to 37% at the end of oogenesis). FFA are few in very young oocytes but they increase in number gradually up to maturity. Free sterols are only present in a significant quantity at submaturity (up to 13.5% of neutral lipids at 180–200 μm and the rate decreases). The ES rate decreases, it goes from 15.5 to 4% during oogenesis.

Neutral lipids in worms deprived of brain neurohormone

A group of worms (oocytes size—approx. 100 μm) is decerebrated. Deprived of brain neurohormone, the *Perinereis cultrifera* are kept in filtered sea water for two weeks. The importance of neutral lipids has been studied (the results are shown in Table 2).

In the coelomocytes MG + DG and free sterols are as numerous as when in animals with normal cerebral endocrine activity. However, the low rate of FFA and ES is in contrast to the observations reported in Table 1, and TG are numerous: FFA: ES and TG are as numerous as in coelomocytes of worms with an oocyte size of 200 μm.

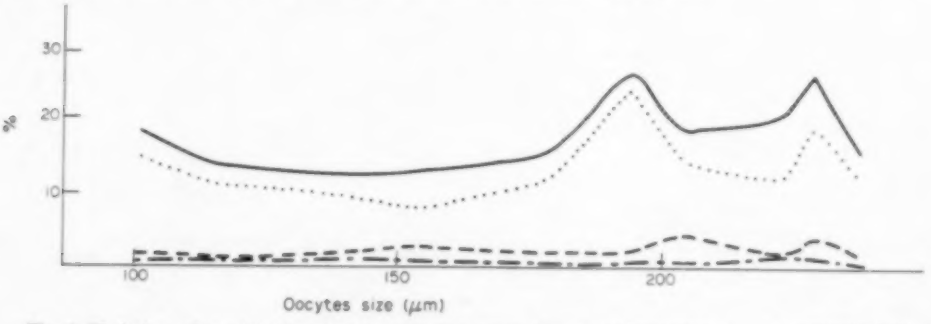

Fig. 1. Variation of total lipid content in coelomic cavity (——), coelomocytes (– – – –), coelomic fluid (— — — —), and oocytes (------) of *Perinereis cultrifera* (percent distribution on a dry weight basis).

Table 1. Variation in the content of lipid classes in the three coelomic constituents of *Perinereis cultrifera*

Lipid class*	Genital stage (oocytes size) 100–120 c.	cf.	o.	140–160 c.	cf.	o.	180–200 c.	cf.	o.	200–220 c.	cf.	o.	220–240 c.	cf.	o.
MG + DG	7.5	29	3	3.5	15	6.5	7	14	9	1	14	2	5	13	2
TG	31	1	77	47	12	68	29	17	48	71	5	57.5	57	6	37
FFA	19.5	44.5	1.5	27.5	28	5.5	33	24.5	21	24	18	20.5	13.5	14	51.5
S	3	3	3	12	14	9	17	12.5	13	3	25	10	23.5	30	5.5
ES	39	22.5	15.5	10	31	11	14	32	9	1	38	10	1	37	4
Total lipid class	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

Mean values of percentages of neutral lipids in coelomocytes (c.), coelomic fluid (c.f.) and oocytes (o.) for three groups of three annelids in the same genital state.
*MG + DG—monoacylglycerols + diacylglycerols; TG—triacylglycerols; FFA—free fatty acids; s—free sterols; ES—esterified sterols.

In the oocytes, after elimination of the hormone, we observe no variation in the rate of acylglycerols and FFA. On the contrary the ES rate decreases and the S rate increases, to reach the same level as that observed at the end of oogenesis in natural conditions. In oocytes the changes that are found in free sterols are opposite to the changes in the esterified sterols and for that reason these changes may be attributed to hydrolytic reactions. In the coelomocytes we do not observe the same phenomenon.

DISCUSSION

Our data show a high level of the fraction MG + DG in coelomic fluid; about 30% of neutral lipids in young worms and about 15% during genital submaturity and maturity. These results are consistent with those obtained in *Nereis virens* by Pocok *et al.* (1969) and in haemolymph of insects where the study of the mechanism of lipid transport has shown that the glycerides released from the fat body are almost all in the form of diacylglycerols (Chino *et al.*, 1965, 1969; Gilbert and Chino, 1974; Engelmann, 1979; Mundall and Law, 1979). On the contrary these diacylglycerols are few in the coelomocytes and in the oocytes of *Perinereis cultrifera*, however their rate increases in oocytes at the beginning of submaturity, then decreases.

The triacylglycerols vary greatly in number but whereas their rate increases in coelomocytes it decreases in oocytes. In coelomic fluid their rate increases in worms of oocyte size from 100 to 200 μm, and then decreases. Free fatty acids are always present in coelomic fluid and coelomocytes, but not in oocytes, except in genital maturity; there are more in oocytes whereas there are fewer in coelomocytes.

We observe that the variations of FFA in cells correspond to opposite changes in the level of TG; it may be postulated that TG are accumulated in coelomocytes and oocytes, and then utilized as an energy source during oogenesis. Experimental elimination of brain activity stimulates acylglycerol storage by coelomocytes but not in oocytes. It may be supposed that the brain hormone plays a role in lipid metabolism of coelomocytes.

Free sterols increase in number in the same way in the coelomic fluid and in the coelomocytes. However, they first appear in coelomocytes. Their rate increases in oocytes up to submaturity (about 200 μm) then decreases. The rate of esterified sterols is high in coelomic fluid but it decreases rapidly in coelomocytes during oogenesis. However, the rate is never high in oocytes and decreases until maturity. In hormone-free experimental conditions there is a considerable diminution of ES in coelomocytes and oocytes. When the ES rate decreases in coelomocytes the rate of free sterols does not increase. Considering the high level of ES in coelomic fluid of normal worms it may be supposed that ES are released by coelomocytes. This phenomenon might be under neuroendocrine control.

Our knowledge about sterol metabolism in annelids is still scant: Voogt (1974a,b) and Ballantine *et al.* (1978) have investigated their composition and capacity of synthesizing sterols, Dales (1969), however, has indicated the level in S and the ES level in

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Table 2. Percentages of neutral lipids (l) in worms deprived of brain neurohormone (oocyte size—100 μm)

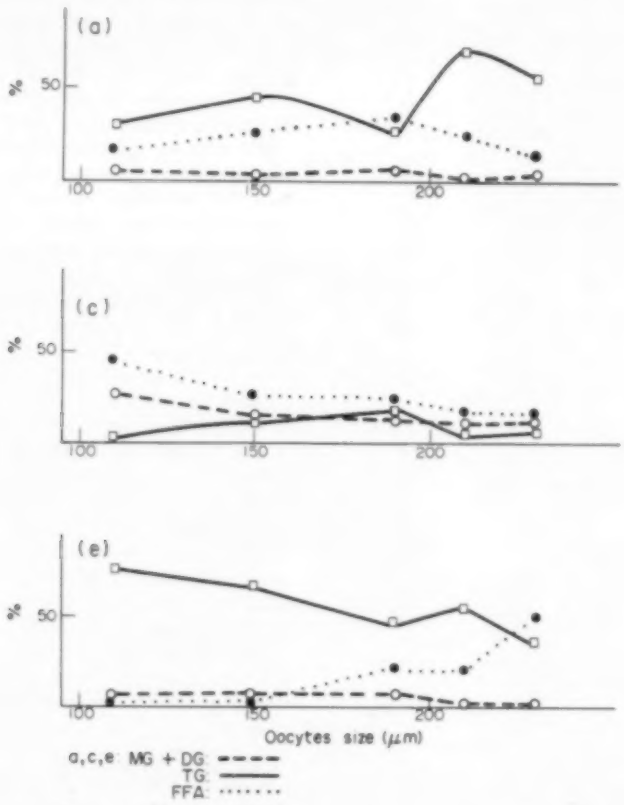
	Genital stage (oocytes size)					
	100 μm + Brain hormone (control)		100 μm − Brain hormone		200–220 μm (Control)	
Lipid class*	c.	o.	c.	o.	c.	o
MG + DG	7.5	3	10.5	0.5	1	2
TG	31	77	67	89	71	57.5
FFA	19.5	1.5	1	0.5	24	20.5
S	3	3	1	9	3	10
ES	39	15.5	20.5	1	1	10
Total lipid class	100	100	100	100	100	100

Mean values of neutral lipids in coelomocytes (c.) and oocytes (o.) for two groups of three annelids.
*MG + DS—monoacylglycerols + diacylglycerols; TG—triacylglycerols; FFA—free fatty acids; S—free sterols; ES—esterified sterols.

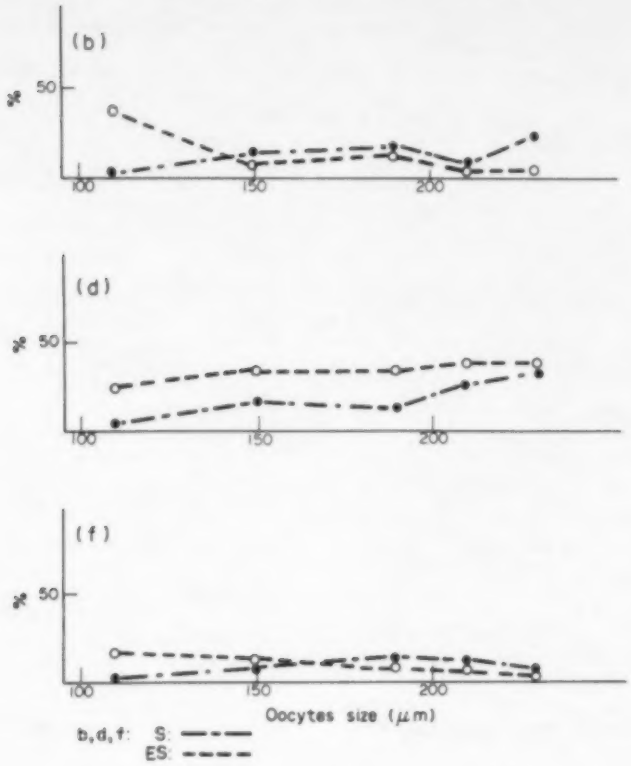
annelids. Our results may lead us to suppose that coelomocytes play an important part in sterol metabolism and a possible role in oogenesis of Polychaetes. Therefore the feed-back substance originating from the oocytes (Cardon and Porchet, 1973) may be able to have a sterol component.

Fat transport through the coelomic fluid and the role of lipids in oogenesis have not been investigated in polychaetes. However, the acylglycerol composition of coelomocytes and coelomic fluid does not differ significantly from that of the fat body and haemolymph of insects. Moreover, neutral lipid composition is under hormonal control, coelomocytes of young worms decerebrated have almost the same composition in neutral lipids as in mature animals.

Finally, electron microscopy revealed that eleocytes of Nereidae are similar to the fat body of insects. Fat body cells of vitellogenic females contain the cytoarchitecture characteristic of cells engaged in massive protein synthesis, namely, a well developed rough endoplasmic reticulum (Hagedorn *et al.*, 1973). In the same way Dhainaut (1966) clearly demonstrated in *Nereis pelagica* similar fine structural changes in sexually mature worm eleocytes. This evolution is more obvious in worms deprived of the brain hormone.



Figs 2(a), (c) and (e). Variations in lipid classes in coelomocytes ((a) and (b)), coelomic fluid ((c) and (d)) and oocytes ((e) and (f)) of *Perinereis cultrifera*. MG + DG, mono and diacylglycerols; TG, triacylglycerols; FFA, free fatty acids, S, sterols; and ES, esterified sterols.



Figs 2(b), (d) and (f). See legend to Figs 2(a), (c) and (e).

What is the role of coelomocytes in acylglycerols synthesis and in their release into coelomic fluid? It may be supposed that coelomocytes play a role in lipoprotein synthesis, exported into the coelomic fluid from where the lipoprotein is taken up by the growing oocytes. Knowledge of the lipid transport system in animals has advanced in recent years, but data on the soluble lipoproteins of species belonging to that vast part of the animal kingdom constituted by the invertebrates is limited to members of phylum Arthropoda. Indeed, the only investigations concern two of the four classes of arthropods, namely crustaceans and insects (see Chapman, 1980, for review). At present in our laboratory we are investigating the means of transport of lipids in coelomic fluid of *Perinereis cultrifera* using radiolabelled elements.

Immunological studies have demonstrated an antigenic identity between coelomocytes, coelomic fluid and oocytes (Dhainaut *et al.*, 1980a,b). Fischer (1979) has shown a vitellin-like antigen in the coelomic fluid of maturing females of *Nereis virens*. But if the transfer of nutrients occurs between eleocytes and oocytes it is in a low mol. wt form, for there is no evidence from electron microscopy to suggest that the oocytes take up yolk protein(s) from the coelomic fluid, during vitellogenesis (Eckelbarger, 1975; Dhainaut, 1970).

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COLONIC ABSORPTION AND SECRETION OF FLUIDS, ELECTROLYTES AND ORGANIC ACIDS IN EAST AFRICAN WILD RUMINANTS

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Abstract—1. In sixteen species of wild ruminants colonic absorption of fluids averaged 48% of the fluids entering the large bowel. Values ranged from 26% (steenbok) to 65% (gerenuk).

2. Absorption of sodium, potassium, chloride and organic acids were variable between species.

3. Colonic absorption of potassium ions were found to be related to diet selection and body weight of the animals.

4. Colonic absorption of volatile fatty acids were significantly greater in browsers (28.4 mmol/l) than grazers (9.6 mmol/l).

5. The African buffalo are the only species of wild ruminants not forming a faecal pellet; colonic functions were not different from those species forming faecal pellets.

INTRODUCTION

Studies of digestive physiology and metabolism of wild ruminants are noticeably limited (Kay *et al.*, 1980; Hoppe *et al.*, 1977a,b; Maloiy *et al.*, 1982; Clemens and Maloiy, 1983; Clemens *et al.*, 1983). Furthermore, the majority of these investigations are restricted to studies of reticulo-rumen functions.

Prior studies of the caecum-colon of wild ruminants relate that: fermentive digestion continues within the large bowel; lower bowel fermentation values appear related to both diet selection and to body weight; osmotic balance is maintained by the inorganic electrolytes and fermentative endproducts; and conservation of body fluids and faecal water loss are prominant functions of the colon (Skadhauge *et al.*, 1980; Maloiy *et al.*, 1982; Clemens and Maloiy, 1983; Clemens *et al.*, 1983). Several questions remain unanswered including: the relationship of diet and body weight to the colonic absorptive process; the fluid, inorganic electrolyte, and fermentative endproduct inter-relationship in the absorptive process; and the relative performance of various colonic segments in the absorptive-secretory process. The present study addresses these questions.

METHODS AND MATERIALS

Fifty-one adult, male animals representing 16 species of East African wild ruminants were used in the study. These include: five Kirk's dik-dik (*Madoqua kirki*), two suni (*Nesotragus moschatus*), three giraffe (*Giraffa camelopardalis*), three gerenuk (*Litocranius walleri*), three eland (*Taurotragus oryx*), four Grant's gazelle (*Gazella granti*), two steenbok (*Raphicerus campestris*), four impala (*Aepyceros melampus*), four Thomson's gazelle (*Gazella thomsoni*), three African buffalo (*Bubalus caffer*), two waterbuck (*Kobus ellipsi prymnus*), three wildebeest (*Connochaetes taurinus*), three hartebeest (*Alcephalus buselaphus*), three topi (*Damaliscus lunatus*), three mountain reedbuck (*Redunca fulvorufula*), and four oryx (*Oryx gazella*). All animals were collected from their natural habitat in conjunction with wildlife management programs. Field analyses and sample collection were begun immediately after sacrifice and generally completed within one hour after death of the animal. Methods of sample collection and laboratory analysis were reported earlier (Clemens and Maloiy, 1983).

Estimates of colonic absorption and secretion of fluids, electrolytes and organic acids were derived by the methods of Staaland (1975). Since organic acids, volatile fatty acids and lactic acid, may be produced and utilized in gut or its tissues, estimates of the colonic flux represent disappearance (absorp-

Table 1. Wild ruminant species categorized according to major feeding group, sub-feeding group and adult weight range*

- I. Browsers
  - a. Fruit and dicotyledon browsers
    - i. Krik's Dik-Dik (5–6 kg)
    - ii. Suni (5–7 kg)
  - b. Tree and shrub browsers
    - i. Giraffe (500–750 kg)
    - ii. Gerenuk (40–51 kg)
- II. Intermediate Feeders
  - a. Prefers browse
    - i. Eland (400–650 kg)
    - ii. Grant's gazelle (46–64 kg)
    - iii. Steenbok (8–11 kg)
  - b. Prefers grasses
    - i. Impala (53–71 kg)
    - ii. Thomson's Gazelle (22–25 kg)
- III. Grazers
  - a. Fresh grass grazers
    - i. African Buffalo (600–850 kg)
    - ii. Waterbuck (192–286 kg)
    - iii. Wildebeest (171–242 kg)
  - b. Roughage grazers
    - i. Hartebeest (116–160 kg)
    - ii. Mountain Reedbuck (23–28 kg)
    - iii. Topi (111–147 kg)
  - c. Dry region grazers
    - i. Oryx (168–209 kg)

*Classifications according to R. R. Hofmann (1973).

Table 2. Mean (±SEM) values for apparent colonic absorption and secretion of fluids, electrolytes, and organic acids for sixteen species of wild ruminants*

Species and colonic segment	Digesta component					
	Water (ml/100 ml)	Sodium (mg/100 ml)	Potassium (mg/100 ml)	Chloride (mg/100 ml)	VFAs (mmol/l)	LA (mmol/l)
Kirk's dik-dik						
Proximal colon	−0.5	5.2	6.0	1.0	1.3	0.2
	(6.1)	(4.9)	(4.9)	(0.6)	(2.6)	(0.1)
Distal colon	42.1	23.8	34.4	9.2	15.5	0.1
	(5.1)	(0.3)	(8.2)	(1.3)	(3.2)	(0.3)
Suni						
Proximal colon	44.2	19.2	41.4	29.2	48.6	0.4
	(4.6)	(8.8)	(21.0)	(16.1)	(40.2)	(0.4)
Distal colon	62.2	26.3	49.2	32.2	66.1	0.4
	(22.6)	(15.6)	(33.2)	(18.6)	(54.5)	(0.4)
Giraffe						
Proximal colon	3.2	34.4	−2.3	0.8	1.6	−0.2
	(21.8)	(39.6)	(9.6)	(1.0)	(3.4)	(0.4)
Distal colon	50.6	49.2	31.4	11.6	27.7	0.2
	(10.5)	(34.2)	(8.6)	(1.8)	(14.4)	(0.2)
Gerenuk						
Proximal colon	5.5	1.4	−14.1	−7.6	0.6	−2.2
	(6.1)	(1.8)	(10.3)	(9.7)	(2.6)	(0.3)
Distal colon	65.1	37.9	4.5	11.1	19.1	0.1
	(14.9)	(16.0)	(10.5)	(2.6)	(22.2)	(0.3)
Eland						
Proximal colon	24.4	5.9	13.8	3.0	5.5	0.1
	(3.8)	(3.8)	(3.4)	(0.1)	(1.4)	(0.1)
Distal colon	37.5	8.8	25.8	7.2	18.6	0.1
	(3.2)	(4.2)	(10.7)	(1.2)	(2.0)	(0.1)
Grant's gazelle						
Proximal colon	24.5	22.2	5.0	0.0	19.1	0.9
	(1.3)	(5.8)	(1.2)	(0.0)	(4.1)	(0.8)
Distal colon	55.7	42.4	9.3	0.1	33.4	1.5
	(5.3)	(8.9)	(0.5)	(0.0)	(4.4)	(1.2)
Steenbok						
Proximal colon	3.2	6.2	6.0	3.0	10.0	−0.8
	(1.4)	(4.4)	(7.8)	(3.3)	(5.8)	(0.4)
Distal colon	26.4	9.4	18.0	9.6	22.6	−0.6
	(2.1)	(0.4)	(5.0)	(2.4)	(7.8)	(0.2)
Impala						
Proximal colon	9.8	10.3	1.9	4.0	0.1	0.1
	(7.8)	(7.4)	(1.5)	(1.3)	(1.5)	(0.1)
Distal colon	38.8	20.9	15.9	12.4	17.3	−0.6
	(8.4)	(4.6)	(6.4)	(2.9)	(8.7)	(0.5)
Thomson's gazelle						
Proximal colon	7.8	3.9	1.4	−1.8	8.4	−0.9
	(13.1)	(6.4)	(1.0)	(0.9)	(2.8)	(0.4)
Distal colon	59.2	32.8	28.2	16.6	30.6	0.5
	(5.0)	(7.2)	(3.9)	(1.2)	(3.0)	(0.3)
African buffalo						
Proximal colon	8.4	10.7	−5.6	−1.1	12.8	0.0
	(0.6)	(12.6)	(11.8)	(11.1)	(19.0)	(0.1)
Distal colon	29.5	31.4	1.8	6.6	19.6	−0.1
	(8.7)	(7.0)	(2.9)	(0.4)	(1.0)	(0.0)
Waterbuck						
Proximal colon	30.4	30.6	−0.4	11.6	13.9	0.1
	(7.0)	(9.7)	(1.4)	(0.8)	(0.2)	(0.1)
Distal colon	44.0	36.4	5.9	15.6	20.1	0.0
	(8.7)	(7.0)	(2.9)	(0.4)	(1.0)	(0.0)
Wildebeest						
Proximal colon	14.3	−0.1	10.4	1.3	3.4	0.1
	(23.0)	(3.8)	(10.7)	(5.0)	(6.5)	(0.1)
Distal colon	31.6	−5.8	14.9	5.3	5.8	0.1
	(1.9)	(5.8)	(6.4)	(2.3)	(4.3)	(0.1)
Hartebeest						
Proximal colon	22.6	8.1	4.1	13.1	1.0	0.1
	(4.2)	(2.3)	(0.8)	(2.7)	(0.1)	(0.1)
Distal colon	66.4	28.2	27.6	27.2	17.6	0.3
	(19.3)	(9.4)	(11.2)	(4.3)	(9.9)	(0.2)
Topi						
Proximal colon	20.5	14.9	4.3	2.5	5.0	0.1
	(4.9)	(3.0)	(1.4)	(0.1)	(0.8)	(0.1)
Distal colon	57.3	31.4	10.4	7.6	11.4	0.2
	(3.1)	(2.6)	(2.3)	(4.3)	(2.7)	(0.1)
Mountain reedbuck						
Proximal colon	17.8	9.5	9.3	1.9	16.3	0.2
	(2.4)	(4.3)	(2.8)	(2.8)	(4.6)	(0.2)
Distal colon	56.4	19.9	30.6	19.1	21.7	0.2
	(6.4)	(5.2)	(4.2)	(2.5)	(0.4)	(0.2)
Oryx						
Proximal colon	11.3	20.4	0.2	4.3	11.0	−0.1
	(9.5)	(10.0)	(2.5)	(2.3)	(3.8)	(0.1)
Distal colon	38.0	42.4	6.3	12.2	24.9	−0.3
	(9.5)	(20.1)	(5.6)	(1.9)	(4.2)	(0.2)

*Positive values represent net absorption (disappearance), negative values represent net secretion (appearance).

tion plus utilization) and appearance (secretion plus production) of these acids. Body weights and food preferences of each species were taken as those reported by Hofmann (1973) (Table 1).

Data were subject to analysis of variance, Duncans Multiple Range test, and regression analysis for determination of significant differences (Steel and Torrie, 1960).

RESULTS

The net absorption–secretion of fluids and electrolytes, and the net appearance and disappearance of organic acids within the proximal and distal colon are presented in Tables 2–4. Approximately 15% of the fluids entering the large bowel were absorbed in the proximal colon, while an average of 45–50% were absorbed within or before the distal colonic measurement. Values ranged from a low of 26% (steenbok) to 65% (gerenuk). When considering the major feeding groups (Table 3), browsers had somewhat greater fluid absorption values than did intermediate feeders and grazers. However, these effects were not significantly different and were primarily due to the greater fluid absorption values observed for the gerenuk. There were no measurable relationship between fluid absorption and body weight of the animal (Table 4).

The movement of sodium, potassium and chloride ions were variable between species. Sodium flux values

Table 3. Mean (±SEM) values for apparent colonic absorption and secretion of fluids, electrolytes, and organic acids for the major and sub-feeding groups of wild ruminants*

Food selection	Digesta component					
	Water (ml/100 ml)	Sodium (mg/100 ml)	Potassium (mg/100 ml)	Chloride (mg/100 ml)	VFAs (mmol/l)	LA (mmol/l)
Major groups						
Browsers						
Proximal colon	13.8	13.5	9.4	5.7	10.8	−0.1
	(9.5)	(7.6)	(7.0)	(4.7)	(8.8)	(0.3)
Distal colon	51.2	30.8	33.8[a]	14.5	28.4[a]	0.2
	(5.8)	(7.3)	(7.5)	(4.1)	(1.7)	(0.2)
Intermediate						
Proximal colon	14.0	10.6	4.6	1.3	8.8	−0.1
	(4.1)	(3.2)	(1.4)	(0.8)	(2.2)	(0.3)
Distal colon	46.4	26.3	18.8[b]	9.3	25.5[ab]	0.3
	(3.9)	(4.3)	(2.7)	(1.8)	(3.0)	(0.4)
Grazers						
Proximal colon	16.4	13.6	3.6	3.8	9.6	0.1
	(4.4)	(3.3)	(3.3)	(1.6)	(2.4)	(0.1)
Distal colon	44.5	26.1	13.1[b]	12.0	17.4[b]	0.0
	(3.6)	(5.8)	(3.2)	(1.8)	(2.6)	(0.1)
Sub groups						
Fruit and dicotyledon						
Proximal colon	18.0	9.2	16.1	9.0	14.8	0.3
	(12.7)	(4.6)	(8.7)	(6.3)	(12.5)	(0.1)
Distal colon	47.9	24.5	38.6	15.8	30.0	0.2
	(7.1)	(6.0)	(9.6)	(5.9)	(15.3)	(0.2)
Trees and shrub						
Proximal colon	4.0	23.4	−6.2	−2.0	1.3	−0.8
	(12.6)	(25.4)	(6.7)	(2.9)	(2.0)	(0.7)
Distal colon	58.8	45.5	22.5	11.5	24.8	0.1
	(10.2)	(20.1)	(10.2)	(1.1)	(8.8)	(0.1)
Prefers browse						
Proximal colon	19.2	14.1	7.4	1.5	13.4	0.4
	(3.6)	(4.2)	(2.2)	(0.8)	(3.1)	(0.3)
Distal colon	43.8	25.7	15.6	4.2	27.0	0.6
	(5.4)	(7.6)	(3.4)	(1.7)	(3.5)	(0.6)
Prefers grass						
Proximal colon	8.8	7.1	1.7	1.1	4.2	−0.4
	(7.1)	(4.7)	(0.8)	(1.3)	(2.1)	(0.3)
Distal colon	49.0	26.9	22.0	14.5	23.9	−0.1
	(6.0)	(4.6)	(4.2)	(1.6)	(5.0)	(0.3)
Fresh grass						
Proximal colon	17.2	11.7	2.7	3.6	9.1	0.1
	(9.5)	(6.4)	(6.8)	(3.7)	(5.2)	(0.1)
Distal colon	34.5	16.9	8.6	8.6	13.8	0.0
	(3.2)	(9.2)	(5.4)	(2.6)	(5.5)	(0.1)
Roughage						
Proximal colon	19.6	11.6	6.4	3.8	9.3	0.1
	(2.2)	(2.3)	(1.6)	(1.9)	(3.1)	(0.1)
Distal colon	58.2	26.0	21.5	15.3	16.7	0.2
	(3.0)	(3.1)	(4.4)	(3.5)	(2.2)	(0.1)
Dry region						
Proximal colon	11.3	20.4	0.2	4.3	11.0	−0.1
	(9.5)	(10.0)	(2.5)	(2.3)	(3.8)	(0.1)
Distal colon	38.0	42.4	6.3	12.2	24.9	−0.3
	(9.5)	(20.1)	(5.6)	(1.9)	(4.2)	(0.2)

Value within the distal colon with unlike superscripts are significantly different ($P < 0.05$).
*Positive values represent net absorption (disappearance); negative values represent net secretion (appearance).

Table 4. Mean (±SEM) values for apparent colonic absorption and secretion of fluids, electrolytes, and organic acids for seven weight groups of wild ruminants*

Weight group	Water (ml/100 ml)	Sodium (mg/100 ml)	Potassium (mg/100 ml)	Chloride (mg/100 ml)	VFAs (mmol/l)	LA (mmol/l)
Less than 20 kg						
Proximal colon	14.7	8.5	13.8	7.7	13.8	0.0
	(9.9)	(3.6)	(6.9)	(4.9)	(9.6)	(0.2)
Distal colon	43.1	21.2	34.0†	14.4	28.3	0.1
	(6.3)	(5.1)	(8.0)	(4.6)	(11.8)	(0.2)
20 to 50 kg						
Proximal colon	11.2	5.7	2.4	−1.2	10.4	−0.6
	(6.4)	(3.4)	(2.9)	(1.5)	(2.8)	(0.4)
Distal colon	60.1	28.6	26.1	16.9	25.8	0.4
	(3.8)	(4.6)	(4.6)	(1.4)	(2.3)	(0.2)
51 to 100 kg						
Proximal colon	17.2	16.2	3.4	2.0	9.6	0.5
	(5.6)	(4.9)	(1.1)	(1.0)	(4.1)	(0.4)
Distal colon	47.2	31.6	12.6	6.2	25.3	0.5
	(5.6)	(6.2)	(3.2)	(2.7)	(5.4)	(0.7)
101 to 150 kg						
Proximal colon	20.5	14.9	4.3	2.5	5.0	0.1
	(4.9)	(3.0)	(1.4)	(0.1)	(0.8)	(0.1)
Distal colon	57.3	31.4	10.4	7.6	11.4	0.2
	(3.1)	(2.6)	(2.3)	(4.3)	(2.7)	(0.1)
151 to 200 kg						
Proximal colon	12.1	17.9	0.9	6.0	9.0	−0.1
	(9.1)	(8.1)	(2.1)	(2.5)	(3.5)	(0.1)
Distal colon	43.7	39.5	10.6	15.2	23.4	−0.2
	(9.3)	(15.8)	(6.1)	(3.4)	(3.6)	(0.3)
201 to 300 kg						
Proximal colon	20.8	12.2	6.1	5.5	7.6	0.1
	(13.4)	(8.4)	(6.4)	(3.7)	(4.4)	(0.0)
Distal colon	36.5	11.0	11.3	9.5	11.5	0.1
	(4.2)	(11.0)	(4.2)	(2.8)	(4.2)	(0.1)
More than 300 kg						
Proximal colon	12.0	17.0	2.0	0.9	6.6	−0.4
	(7.0)	(12.1)	(7.2)	(3.0)	(5.4)	(0.1)
Distal colon	39.2	29.8	20.0	8.5	22.0	0.1
	(4.8)	(12.3)	(8.4)	(2.2)	(6.7)	(0.1)

*Positive values represent net absorption (disappearance), negative values represent net secretion (appearance).
†Significant regression analysis ($P < 0.02$) for reduced potassium absorption (distal colon) relative to increased body weight.

ranged from a net colonic secretion in the wildebeest (−5.8 mg/100 ml) to 49 mg absorption/100 ml observed in the giraffe. Potassium values were also variable among species (low of 1.8 mg/100 ml in the African buffalo to a high of 49.2 g/100 ml in the suni). Chloride values were the least variable among species and, with the exception of the suni, were within the range of 0–15 mg/100 ml. Of the three electrolytes only potassium showed a significant relationship with the animals' dietary habits (browsers having the greater potassium absorption, 34 mg/100 ml, and grazers the least, 13.1 mg/100 ml). Potassium absorption was also found to be related to the animals body weight, with the lighter animals having the higher absorption.

Volatile fatty acid (VFA) values were equally as variable among species as were the inorganic electrolytes. Distal colonic VFA disappearance values ranged from 5.8 mmol/l (wildebeest) to 66.1 mmol/l (suni). VFA disappearance values were significantly related to diet selection (Table 3). As with the potassium ions, net VFA disappearance was greatest in browsers (28.4 mmol/l) and least in grazers (17.4 mmol/l). Lactic acid values of the colonic contents were generally too low for significant measurements.

DISCUSSION

The mammalian large intestine and rectum have been characterized as the "digestive organs for salt and water conservation" (Skadhauge *et al.*, 1980). Fermentative digestion has also been indicated as a prominent feature of the large bowel (Alexander, 1965; Clemens, 1979; Stevens *et al.*, 1980). Although, in ruminants, colonic bacterial fermentation contribution to fiber digestion is believed limited (Goodall and Kay, 1965; Hoover, 1978; Skadhauge *et al.*, 1980), organic acids are a principle component of the intestinal contents (Maloiy and Clemens, 1980a).

Skadhauge and co-workers (1980) have shown that the amount of dry matter digested within the large bowel of the small ruminant (i.e. dik-dik antelope) is minimal, and that, compared to inert markers, dry matter content is an acceptable tool for calculating fractional absorption rates of fluids and electrolytes. A detailed description of this technique using dry matter as the colonic marker was presented by Staaland (1975).

When considering the large bowel, several investigators have shown prominent differences in the colonic electrolyte flux between the proximal and distal regions (Stevens *et al.*, 1980; Skadhauge *et al.*, 1980; Maloiy and Clemens, 1980b). Within the present group of wild ruminants the spiral colon generally comprised the proximal half and had a fluid to pasty consistency (dry matter range 11–24%). Caecal contents were somewhat more fluid with a dry matter averaging 16% (Clemens and Maloiy, 1983). For these animals the distal colon represents the region of faecal

pellet formation, for all species except the African buffalo. Dry matter consistency within this segment of tract ranged from 23 to 65%, with a mean value of 34%. The African buffalo which does not form a faecal pellet had a mean distal colonic dry matter of 15%.

Stevens and co-workers (1980) described two colonic epithelial transport systems. The first system is sodium dependent in which sodium and VFAs are absorbed at similar rates. This results in the increased movement of fluids from the gut. The sodium-driven fluid recovery system is characteristic of that seen in the distal colon of the pony, colon of the dog and colon of the goat (Stevens *et al.*, 1980). The second system is one in which VFAs are absorbed more readily than sodium ions. In this system VFAs are absorbed in predominantely the undissociated form, resulting in less water absorption from the gut. Such a system is characteristic of that seen in the reticulo-rumen, proximal colon of the pony and colon of the pig. The system allows for the accumulation of fluids and bicarbonates, for bacterial proliferation and for the removal of fermentative endproducts (VFAs). The present study would suggest that East African wild ruminants possess colonic transport systems favorable for fluid recovery within both the proximal and distal colon, and less favorable for fermentative digestion, as evident by the sodium : VFA absorption ratio. The one exception to these findings is the values observed for the suni. In this instance VFAs were absorbed several times more readily than sodium ions. However, potassium absorption is elevated in the suni, relative to other species of wild ruminants, and appears to be the driving force for VFA and water transport.

While the data would suggest that colonic conditions are not highly favorable for fermentative digestion, such actions are occurring within the large bowel of the wild ruminant (Maloiy *et al.*, 1982). VFA formation and removal was an important part of the colonic functions.

The significant values observed for diet selection, potassium absorption and VFA disappearance is in part explainable from the data presented in an earlier publication (Clemens and Maloiy, 1983). In that study, browsers were observed to consume more potassium (reticulo-rumen contents, 99.8 m-equiv/l) than either intermediate feeders (69.9 m-equiv/l) or grazers (42.4 m-equiv/l). The relationship was carried on through the caecal and colonic contents of these animals. Thus, more potassium was available for absorption, possibly accounting for the significant findings. Similarly, colonic VFA concentrations were greater in browsers and intermediate feeders than those observed for grazers (Clemens and Maloiy, 1983).

The one species which may have been expected to, but did not show, a marked difference in colonic functions was the African buffalo. This animal was the only member of the wild ruminants which did not form a faecal pellet. As such it might have been expected that colonic transport systems would favor those retaining fluids within the gut, as was observed for Zebu cattle (Maloiy and Clemens, 1980b). While smaller quantities of fluids were absorbed from the colon of the African buffalo, relative to most other species, the values were not significantly different from several species forming faecal pellets (most notably the wildebeest and steenbok). Again, potassium absorption from the colon of the African buffalo was considerably lower than most other species of wild ruminants and may be the driving force for the reduction in fluid absorption seen in these animals.

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EFFECT OF EXTERNAL SALINITY CHANGE ON THE ADENYLATE ENERGY CHARGE IN THE BRACKISH BIVALVE *CORBICULA JAPONICA*

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Abstract—1. The energy charge in the brackish clam *Corbicula japonica* changed within the range of 0.6–0.8 during exposure to osmotic or anoxic stress.

2. The energy charge elevated transiently at the initial day of hypoosmotic stress and at the third day of hyperosmotic stress. Exposure to anoxia resulted in a small but significant decline in energy charge. The transient elevation during osmotic stress may be a reflection of aerobic metabolism.

3. During exposure to these stresses, activities of key enzymes involved in amino acid metabolism may be regulated by the energy charge.

INTRODUCTION

It is well known that in many aquatic molluscs the concentration of intracellular free amino acids (FAA) changes in response to salinity change in external medium (Virkar and Webb, 1970; Pierce, 1971; Gainey, 1978). The change in FAA concentration is considered to be important in cell volume regulation or isosmotic intracellular regulation during acclimation to salinity change. Although the biochemical mechanism underlying the FAA fluctuation is obscure, glutamate dehydrogenase (GDH) is assumed to be one of the important enzymes involved in FAA accumulation during acclimation to salinity increase (Gainey, 1978; Baginski and Pierce, 1978). We reported previously that GDH activities of the bivalves *Corbicula japonica* and *C. leana* were markedly affected by the adenylate energy charge in reaction mixture (Matsushima and Kado, 1983).

Atkinson (1968, 1977) has proposed that the energy level in the adenylate system can be quantitatively represented by the energy charge (ATP + 1/2 ADP)/(ATP + ADP + AMP) and that activity of the key enzymes involved in energy metabolism and biosynthesis is under control of this parameter. Some enzymes are reported to respond to the energy charge (Atkinson and Walton, 1967; Thompson and Atkinson, 1971), though the response pattern is dependent upon some other factors (Purich and Fromm, 1972, 1973). As a condition influencing the energy charge in intact animals, anoxic stress has been known to reduce its value markedly in some animals (Ridge, 1972; Wijsman, 1976; Ellington, 1981). However, little is known about the effect of osmotic stress on the energy charge. The present study was primarily designed to examine whether the energy charge *in vivo* could account for the physiological significance of the *in vitro* energy charge response of GDH.

In this study, the effect of both hypo- and hyperosmotic stresses on the energy charge of the brackish bivalve, *Corbicula japonica*, was investigated in the context of intracellular osmoregulation mediated by change in FAA pool size. In addition, change in water content of tissue, which is considered to be an index of cell volume regulation, was examined to compute the net quantity of adenine nucleotides excluding the effect of tissue hydration or dehydration.

MATERIALS AND METHODS

Acclimation of animal

Specimens of *Corbicula japonica* (shell length of 2–3 cm) were collected from a brackish river in Hiroshima City, where salinity of water was remarkably changed by tidal cycle. At low tide, osmotic concentrations of water and filtrate of the bottom mud in the habitat were about 100 mOsm and 300–400 mOsm, respectively. The animals were acclimated to 35% sea water (340 mOsm) or to fresh water (3–7 mOsm), which was recirculated and aerated, for more than two weeks at water temperature of 20–26°C. In order to examine the effect of osmotic stress, the animals acclimated to 340 mOsm were transferred to fresh water and the fresh water acclimated animals to 340 mOsm. At specified intervals, concentrations of adenine nucleotides in total tissue (whole animal except for valves and adductors) were determined as described below. This species tolerated both osmotic stresses and survived in the respective media for several months.

For the experiments on anoxic stress, the animals were treated in two ways; the 340 mOsm acclimated animals were transferred to a vessel containing the same medium (6 individuals/l) constantly bubbled with N_2 gas or to a closed vessel containing N_2 gas only without water. Two days after the transfer to the anoxic conditions, concentrations of the adenine nucleotides in total tissue were determined.

In all experiments conducted here, valves were not wedged open. Controls were simultaneously worked up using the animals without stress. Medium for acclimation was prepared by diluting filtered natural sea water with tap water and osmotic concentration of the medium was determined with an osmometer (Advanced, DII).

Analytical reagents

Enzyme products of phosphoglycerate kinase (PGK)–glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mixture, lactate dehydrogenase (LDH), pyruvate kinase (PK) and myokinase (MK), nucleotides of ATP, ADP and AMP for

standards, substrates of glycerate-3-phosphate and phosphoenolpyruvate, and triethanolamine hydrochloride were obtained from Boehringer. Cofactor NADH was purchased from Sigma. All other chemicals used were of reagent grade.

Determination of adenine nucleotides

Tissue extract was prepared by a method similar to that of Wijsman (1976). In brief, excised total tissue was promptly blotted with filter paper and dropped in liquid nitrogen. It took about 1 min from excision to freezing. The frozen tissue (1–2 g) was removed from liquid nitrogen, rapidly weighed to the nearest 0.01 g and homogenized in 5 vol of ice-cold 7% trichloroacetic acid (TCA) using an Ultra-Turrax homogenizer before the frozen tissue melted. The homogenate was centrifuged at 25,000 *g* for 60 min. The supernatant was neutralized with 5 M K_2CO_3 and analysed for ATP, ADP and AMP. Factor of dilution effect of K_2CO_3 was taken into account (1.04). In order to examine the recovery of the three nucleotides in this method, frozen tissue was homogenized together with a standard sample of known concentration of nucleotide mixture. For each nucleotide, a high recovery percentage of almost 100% was obtained.

Concentrations of ATP, ADP and AMP were determined enzymatically by the method of Jaworek *et al.* (1974a,b). Final volume of reaction mixture was 3.0 ml for ATP assay and 2.8 ml for ADP and AMP assay, in which 0.2 ml sample and 20 μl of respective enzyme products were included. Activities of enzymes added to the assay mixture were 8 U of both PGK and GAPDH for ATP determination and 50 U of LDH, 40 U of PK and 35 U of MK for ADP and AMP determination. Decrease in absorbance ($\Delta E_{340} = 6.3 \times 10^3$) by oxidation of NADH was measured using 1 cm quartz cuvettes and a spectrophotometer (Hitachi 100-20) equipped with a thermostatted chamber (30°C). A series of known concentration of nucleotides were assayed in comparison between the presence and absence of TCA. In the presence of TCA, ATP was underestimated as reported by Wijsman (1976). Therefore, inhibition factor was included in the calculation of concentration (1.08). Further, TCA delayed completion of MK reaction for AMP assay, but it did not affect the endpoint. For the experiments on osmotic stress, nucleotide levels were expressed as μmol/g dry wt by including the water content of tissue in the calculation.

Water content

At specified times after the osmotic shock, the animals were removed, and acclimating water on the shell surface was wiped away with tissue paper. Then, valves were pried open by cutting the adductors and the total tissue was excised. The tissue was blotted with filter paper, weighed and dried overnight in a 95°C drying oven. Water content was calculated from the difference between the wet and dry wt and expressed as % of wet wt.

Statistics

Student's *t*-test was used to compare experimental groups with controls and $P < 0.05$ was used as the level of significance.

RESULTS

Volume regulation

Time course of volume regulation was examined by means of the water content in total tissue of the animals exposed to hypo- (Fig. 1a) and hyperosmotic stress (Fig. 1b). As shown in Fig. 1(a), water content of the control animals acclimated to 340 mOsm was 77–78% of wet tissue wt. When the animals were transferred to fresh water, the water content increased remarkably within 5 hr and reached the maximum of 86.4% in 10 hr. Thereafter, the content began to decrease towards the control level; after one day the water content

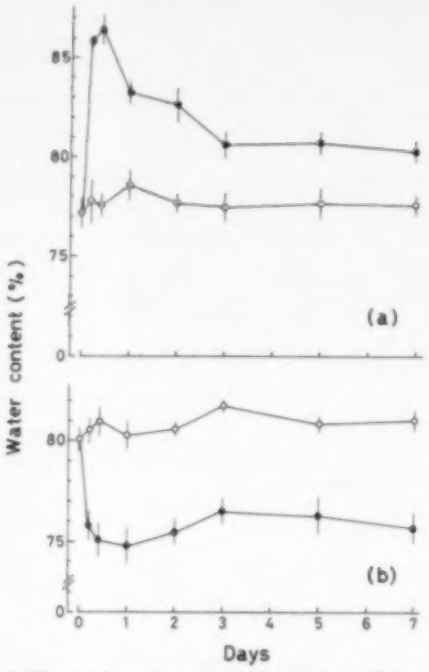

Fig. 1. Changes in water content of total tissue of *Corbicula japonica* during acclimation to hypo- (a) and hyperosmotic (b) stress. (a) Clams acclimated to 340 mOsm and transferred to 340 mOsm (○) or to fresh water (●). (b) Clams acclimated to fresh water and transferred to fresh water (○) or to 340 mOsm (●). Points ± vertical lines are the means ±SE (n = 6–8).

recovered to 83.2% and after three days to 80.6%. This value of 80.6% was not different statistically from that of animals acclimated to fresh water for two weeks ($P > 0.5$), but the water content never returned to the original level of control animals kept in 340 mOsm; it was significantly higher than controls at the 5th day ($0.01 < P < 0.02$) and at the 7th day ($P < 0.01$).

When the animals acclimated to fresh water were transferred to 340 mOsm, they did not show an evident process of volume regulation (Fig. 1b). Water content of the control animals kept in fresh water was maintained within the range of 80.1–81.8% during the experimental period. Upon transfer to 340 mOsm the animal tissue lost water rapidly; after 5 hr the water content decreased significantly to a level lower than that of controls ($P < 0.001$) and reached the lowest level of 74.8% after one day. The water content of the experimental animals was maintained significantly lower than that of controls throughout the experimental period ($P < 0.001$ at each time).

Effect of osmotic stress on the energy charge

In the animals acclimated to 340 mOsm, the energy charge of the total tissue was around 0.6 at each specified time of sampling (Fig. 2). When the animals were transferred to fresh water, the energy charge increased significantly to 0.75 after one day ($P < 0.001$). However, the increase was transient; at the 3rd ($P > 0.5$), 5th ($P > 0.5$) and 7th day ($P > 0.05$) difference in the energy charge between the experimental animals and controls was not significant. This change in energy charge reflected the change in each

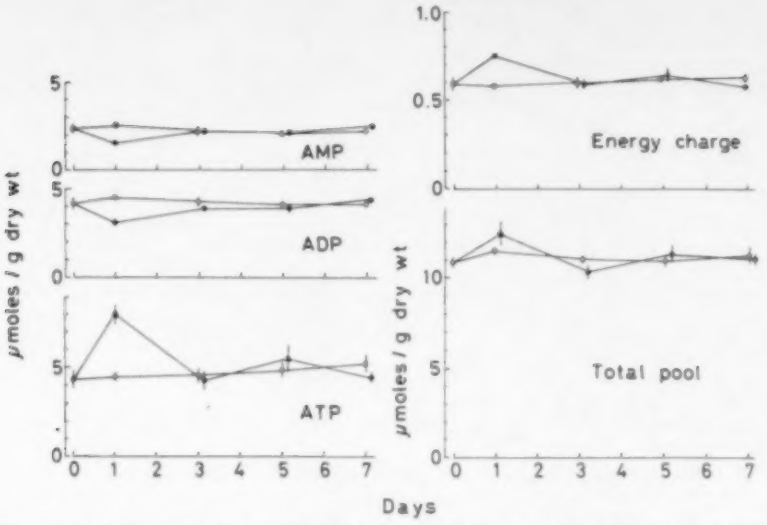

Fig. 2. Changes of adenine nucleotide levels in total tissue of *Corbicula japonica* and the calculated parameter of the adenylate pool during acclimation to hypoosmotic stress. Clams acclimated to 340 mOsm and transferred to 340 mOsm (○) or to fresh water (●). Points ± vertical lines are the means ± SE (n = 5–8). For those points without vertical lines, standard errors are less than the size of the points.

nucleotide; a marked increase (1.8-fold) in ATP accompanied by a decrease in ADP and AMP was observed after one day, but thereafter (3, 5 and 7 days) the level of each nucleotide was nearly the same in the control and experimental animals.

In the reverse osmotic stress, the energy charge of the control groups kept in fresh water was 0.6–0.7 throughout the experimental period (Fig. 3). At the 3rd day after transfer to 340 mOsm, the energy charge of the experimental animals was significantly higher than that of controls ($P < 0.001$), reflecting an increase in ATP and decreases in ADP and AMP. However, this increase was transient; at the 1st ($P > 0.1$), 5th ($P > 0.5$) and 7th day ($P > 0.2$) the energy charge of the experimental animals could not be distinguished statistically from that of the controls. Changes in quantity of ATP were completely reverse to those of ADP and AMP in both hypo- and hyperosmotic acclimation. The values of the energy charge were considerably different from one individual to another, though scarcely below 0.6. At specific times (the initial day of hyperosmotic stress and the 3rd day of hyperosmotic stress), however, most individuals showed high values of the energy charge.

Effect of anoxic stress on the energy charge

The energy charge of the animals which were kept in 340 mOsm sea water gassed with N_2 ($P < 0.02$) or in

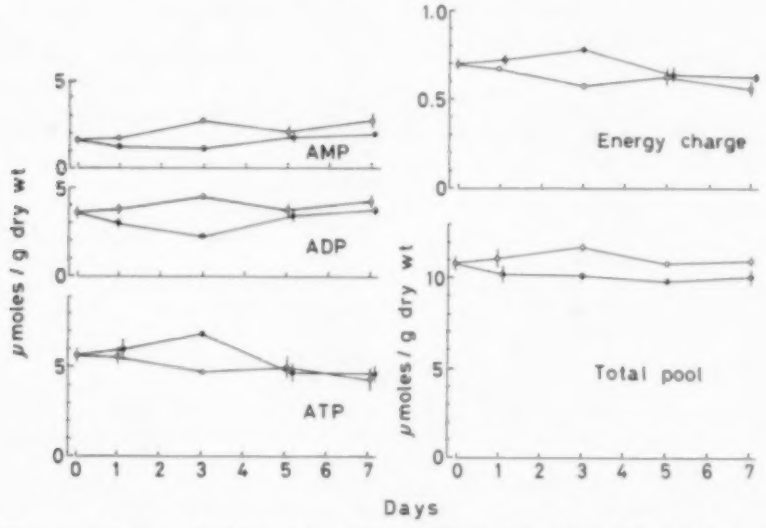

Fig. 3. Changes of adenine nucleotide levels in total tissue of *Corbicula japonica* and the calculated parameter of the adenylate pool during acclimation to hyperosmotic stress. Clams acclimated to fresh water and transferred to fresh water (○) or to 340 mOsm (●). Points ± vertical lines are the means ± SE (n = 5–8). For those points without vertical lines, standard errors are less than the size of the points.

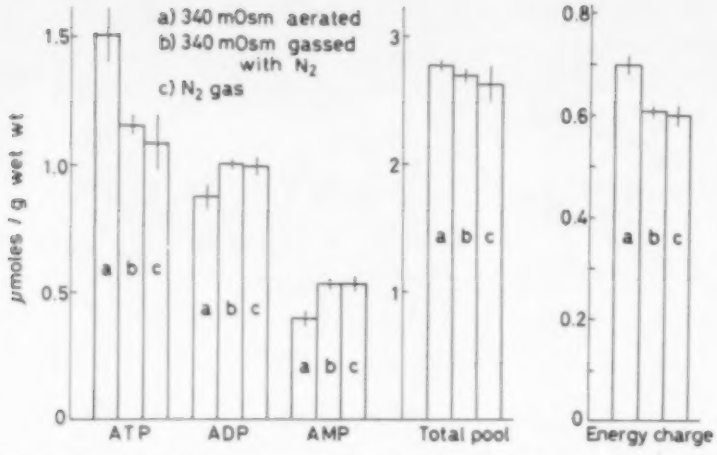

Fig. 4. Effect of anoxic stress (48 hr) on concentration of adenine nucleotides in total tissue of *Corbicula japonica* and the calculated parameter of the adenylate pool. Clams acclimated to 340 mOsm and transferred to (a) 340 mOsm aerated; (b) 340 mOsm gassed with N_2; and (c) N_2 gas only without water. Columns ± vertical lines are the means ±SE (n = 5–6).

N_2 gas without water ($P < 0.01$) for two days was slightly but significantly lower than that of the control animals acclimated to well aerated 340 mOsm sea water (Fig. 4). The two kinds of anoxic stresses caused to the same extent a decrease in concentration of ATP and increase in ADP and AMP. The decrease in ATP was completely accounted for by the sum of increase in ADP and AMP, and this resulted in unchanged pool size of total adenylates ($P > 0.5$). Although some of the animals placed in 340 mOsm gassed with N_2 were observed to open their valves remarkably during the course of experiment, they responded well to mechanical stimulation.

DISCUSSION

Concentration of total adenylate pool in total tissues of *Corbicula japonica* was quite similar to that in total tissue, hepatopancreas and mantle of *Mytilus edulis* (Wijsman, 1976), but lower than the concentration in adductors of *M. edulis* (Wijsman, 1976), *Chlamys operculatis* (Grieshaber, 1978) and *Lima hians* (Gade, 1981). Beis and Newsholme (1975) investigated the contents of adenine nucleotides in muscle tissues from many animal species of vertebrates and invertebrates and found that the lowest concentrations of ATP and total adenylates were determined in the molluscan muscles except for *Pecten maximus*. The high concentration of ATP and total adenylates in adductor muscles of *Chlamys*, *Lima* and *Pecten* were related to swimming behavior of these species.

The energy charge values of *C. japonica* ranged from 0.6 to 0.8 during acclimation to salinity change. This range of energy charge was similar to that determined for *M. edulis* (Wijsman, 1976) and the sea anemone *Bunodosoma cavernata* (Ellington, 1981), which were exposed to anoxic stress. However, the control group of *C. japonica* without stress exhibited lower energy charge (0.6–0.7) than that of the above species. Many species of intertidal bivalves are known to depend conspicuously upon anaerobic metabolism which produces alanine as one of the major end-products (Hochachka and Mustafa, 1972; de Zwaan and Wijsman, 1976; de Zwaan, 1977). Mangum and Burnett (1975) observed that *Rangia cuneata* and *Mya arenaria* extracted only a small part of oxygen from water which was ventilated by the animals under normoxic condition. The normoxic anaerobiosis may account for the low level of the energy charge observed in control animals of *C. japonica*. In fact, the energy charge of the animals exposed to anoxic stress fell by only 0.1 and this decrement by anoxic stress was much smaller than that determined for the foregoing species of the sea mussel and sea anemone. *Corbicula japonica* inhabits the bottom mud of river near the estuary, whose environment is labile in salinity, temperature and oxygen concentration and even ebbs away at low tide. As pointed out by Mangum and Burnett (1975), adaptation to anaerobiosis may be the result that intracellular osmoregulation mediated by free amino acids would have been more serious problem to these animals than efficiency of energy production.

The energy charge of *C. japonica* elevated transiently at the initial day of hypoosmotic stress but at the third day of hyperosmotic stress. Such a different time course between hypo- and hyperosmotic stress was observed in other responses of this species (Matsushima, 1982). That is, during acclimation to salinity decrease the osmotic pressure of the mantle fluid and concentration of ninhydrin positive substances (NPS) in tissue were decreased within a day to the level of fresh water acclimated animals. During acclimation to salinity increase, on the other hand, it took 3–5 days for those values to increase to almost the level of the animals acclimated to high salinity. Thus, the change in energy charge presented here is closely related to both organismal and cellular responses to osmotic stress. Since the change in osmotic concentration of the mantle fluid reflects the ventilatory activity of the clam, the time required for osmotic equilibrium between the mantle fluid and external medium would be related to the resumption of oxygen uptake after osmotic shock. In fact, *Rangia cuneata* resumed oxygen uptake more rapidly after hypo-

osmotic transfer than after hyperosmotic transfer (Henry and Mangum, 1980; Henry *et al.*, 1980). From this point of view, the transient conversion from anaerobic metabolism to aerobic one appears to account for the elevation in energy charge during osmotic stress.

According to the theory of Atkinson (1968), the activity of key enzymes related to energy metabolism and biosynthesis is affected by energy charge within the range of change in *C. japonica* after osmotic shock. Mustafa and Hochachka (1971) have reported that pyruvate kinase of *Crassostrea gigas*, the enzyme that participates in alanine production by catalyzing conversion from phosphoenolpyruvate to pyruvate, is inhibited by ATP. Further, we have found that glutamate dehydrogenase (GDH) of *C. japonica* responds typically to the energy charge; reduction in the energy charge in reaction mixture including 3 mM total adenylate pool resulted in activation of the GDH (Matsushima and Kado, 1983). It can be considered, therefore, that activity of both enzymes *in vivo* would be closely related to change in the energy charge during acclimation to salinity change. Since alanine is the major component of FAA pool in *C. japonica* (Matsushima *et al.*, 1982), change in the energy charge during osmotic stress may play an important role in intracellular osmoregulation by controlling these enzyme activities.

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ENHANCEMENT OF TASTE RESPONSES TO ACIDS BY CALCIUM IONS

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Abstract—1. The frog taste nerve responses to HCl and acetic acids were greatly enhanced by increasing calcium concentration in a solution to which the tongue had adapted when the lingual artery was perfused with Ringer solution.
2. Enhancement was not seen in the responses to other taste stimuli.

INTRODUCTION

Sour taste is one of the fundamental tastes (Pfaffmann, 1971) and is induced by acids. Beidler (1971) pointed out that the taste equation (Beidler, 1954) can be applied to the acid response, which suggested that the binding of acids to the taste receptor membrane leads to generation of the acid response. Although the magnitude of the taste response to acids depends partly on species of acid anions (Beidler, 1967), concentration of protons mainly determines the magnitude of the acid response. These results suggested that the initial event of the acid reception is the binding of proton to the taste receptor membrane (Beidler, 1967, 1971). The binding of protons leads to depolarization of taste cells (Tateda and Beidler, 1964; Ozeki and Sato; Akaike *et al.*, 1976; Sato, 1980), which induces the impulses in the taste nerves.

Although protons have the simplest structure among all the taste stimuli, only limited information on the receptor site for protons has been obtained. Koyama and Kurihara (1972) pointed out the possibility that the phospholipids in the taste receptor membranes are the receptor site for protons. Hiji (1975) demonstrated that the rat taste response to HCl is not affected by the action of proteolytic enzymes applied to the tongue surface, while the response to sucrose is eliminated by the enzymes. In spite of these studies, the actual molecular properties of the receptor site for acids are still unknown. In this paper, we will show that the responses of the frog taste nerve to acids largely depend on calcium concentration in a solution which adapts the tongue under the stated conditions and will discuss the role of Ca^{2+} ions in acid reception.

MATERIALS AND METHODS

Recording of the taste nerve responses

Adult bullfrogs, *Rana catesbeiana*, were used in these experiments. The dissection of the glossopharyngeal nerve and the recording of the neural activity were carried out as described previously (Nagahama *et al.*, 1982). After a frog was anesthetized with an intraperitoneal injection of urethane solution (about 300 mg/100 g body weight), the glossopharyngeal nerve was separated from the surrounding tissues and cut proximally.The tongue was pulled out and fixed with insect pins on a corkboard in an experimental chamber.

The nerve impulses were amplified with an a.c.-amplifier and integrated with an electronic integrator with a time constant of 0.3 sec.

Perfusion of the lingual artery

Perfusion of the lingual artery with normal Ringer solution (112 mM NaCl, 3.4 mM KCl, 3.6 mM $MgSO_4$, 2.5 mM $CaCl_2$, 2.5 mM $NaHCO_3$, pH 7.2) was also carried out as described previously (Nagahama *et al.*, 1982). A polyethylene tube was cannulated into the lingual artery and Ringer solution containing sodium heparine of 10 U/ml was perfused through the tube into the artery using a peristaltic pump at a rate of 0.01 ml/sec. The perfused solution was drawn out from the vein at the bottom of the tongue. After the blood had been completely replaced with Ringer solution, stimulating solutions were applied to the tongue.

Chemical stimulation

After the tongue was allowed to adapt to Ringer solutions containing Ca^{2+} of varying concentrations for 15 min, stimulating solutions were applied at a flow rate of 1.7 ml/sec. Essential differences in the effect of Ca^{2+} ions on the acid responses were not found between tongue adaptation times of 5 and 15 min. Stimulating solutions of $CaCl_2$, $MgCl_2$ and NaCl were prepared by dissolving the chemicals in distilled water. In order to suppress the water response, quinine hydrochloride, HCl and acetic acid were dissolved in 5 mM KCl solution (Miyake *et al.*, 1976).

All experiments were carried out at 20°C.

RESULTS AND DISCUSSION

Figure 1A shows the summated responses of the frog glossopharyngeal nerve to 0.1 mM HCl after the tongue had previously adapted to Ringer solutions containing 0, 2 and 10 mM $CaCl_2$. The magnitude of the responses to HCl is practically independent of the calcium concentration in the adapting solution. On the other hand, the response to HCl depends largely on the calcium concentration when the lingual artery is perfused with normal Ringer solution, as shown in Fig. 1B—the response to HCl is increased with increasing the calcium concentration in the adapting solution. In the following experiments, stimulating solutions were applied to the tongue when the lingual artery was perfused with normal Ringer solution.

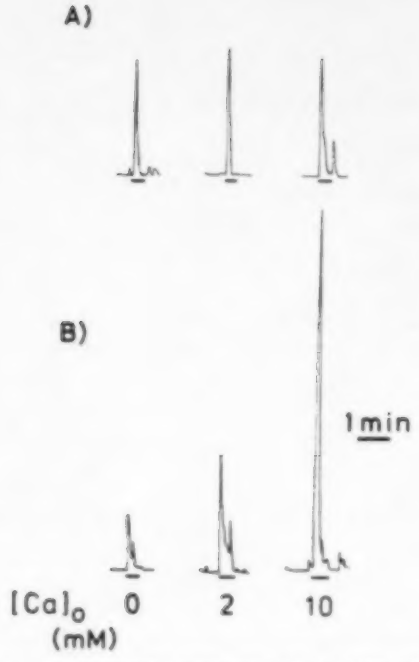

Fig. 1. The summated responses of the frog glossopharyngeal nerve to 0.1 mM HCl. The frog tongue was allowed to adapt to Ringer solution containing 0, 2 and 10 mM $CaCl_2$ for 15 min and then 0.1 mM HCl was applied. Bars at the bottom of each record represent the duration of stimulation. A: The lingual artery was not perfused. B: The lingual artery was perfused with normal Ringer solution as described previously (Nagahama *et al.*, 1982).

Figure 2A shows the magnitude of the peak responses to 0.1 mM HCl, 0.1 mM acetic acid, 1 mM $CaCl_2$, 400 mM NaCl, 0.1 mM quinine hydrochloride and distilled water as a function of the calcium concentration in the adapting solution. Here the magnitude of response to each stimulus when the calcium concentration is zero is taken as unit. The responses to HCl and acetic acid are greatly increased with increasing calcium concentration in the adapting solution and reach the saturation level around 10 mM $CaCl_2$. On the other hand, the responses to $CaCl_2$, NaCl, quinine hydrochloride and distilled water are practically independent of the calcium concentration in the adapting solution. Figure 2B shows the magnitude of the responses to 0.1 mM HCl and 1 mM $CaCl_2$ as a function of the magnesium concentration in the adapting solution. Mg^{2+} ions in the adapting solution exhibit no enhancing effect on the responses to both HCl and $CaCl_2$. Changes in the concentration of NaCl or KCl in the adapting solution containing no Ca^{2+} ions did not practically affect the acid response.

Figure 3 shows the magnitude of the response to HCl as a function of pH of the adapting solutions which contained 2 and 10 mM $CaCl_2$. The responses increase with decreasing pH but decrease below pH 3.5. Application of HCl solution below pH 3.5 brought about partly irreversible effect on the receptors. Figure 3 shows that an increase in the calcium concentration from 2 to 10 mM does not affect the threshold concentration for HCl but leads to an enhancement of the response at each pH by a factor of about 3.

The present results show that the responses to acids are largely increased with increasing calcium concentration in the adapting solution only when the lingual artery is perfused with the artificial solution. During the course of preparation of this manuscript, we noticed that T. Sato (personal communication) also observed (independently) the enhancing effect of Ca^{2+} ions on the acid response. The adaptation of the frog tongue to a solution containing only Ca^{2+} ions instead of Ringer solution, led to the enhancement of the acid response when the lingual artery was not perfused with the artificial solution. These results, together with the present ones showing that the adaptation of the tongue in Ringer solution did not lead to the enhancement of the acid response under

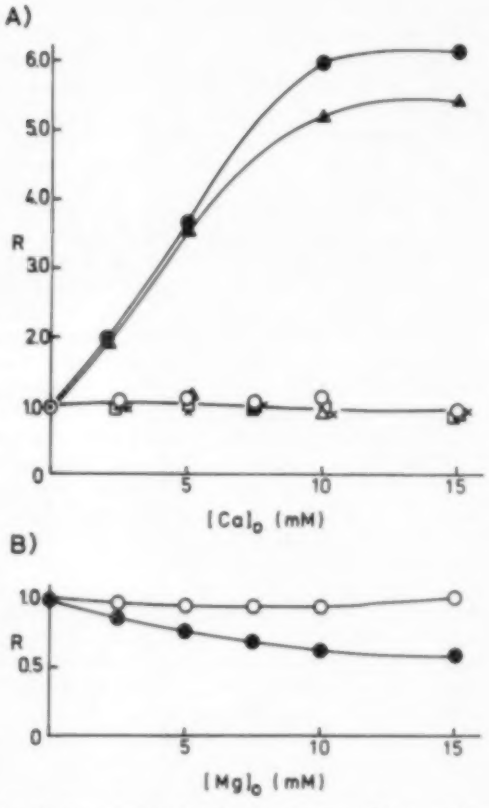

Fig. 2. The magnitude of the responses (R) to various stimuli as a function of the calcium concentration (A) and the magnesium concentration (B) in Ringer solutions to which the frog tongue had adapted. The lingual artery was perfused with normal Ringer solution. The magnitude of the summated response at the peak is taken as the magnitude of the response. The magnitude of the response to each stimulus when the calcium concentration or the magnesium concentration is zero is taken as unit in the ordinate. Each point in the figure represents the mean value of the data obtained from at least three different preparations. ●, 0.1 mM HCl; ▲, 0.1 mM acetic acid; ○, 1 mM $CaCl_2$; △, 400 mM NaCl; □, 0.1 mM quinine hydrochloride; ×, distilled water.

these conditions, suggest that NaCl in normal Ringer solution suppresses the enhancing effects of Ca^{2+} ions on the acid response. On the other hand, NaCl in the Ringer solution does not suppress the enhancing effect when the ligual artery is perfused with the artificial solution as shown in Fig. 1A. Since the ionic composition of the plasma in the blood is considered to be similar to that of normal Ringer solution, it is unlikely that the changes in the ionic composition by replacement of the blood with normal Ringer solution cause the enhancing effect of Ca^{2+} ions in the adapting solution on the acid response. During the perfusion of the lingual artery, the tongue swelled because of the pressure applied through the peristaltic pump and hence conformational change of the receptor membrane seemed to occur, which might lead to an increase in the sensitivity of the acid response to Ca^{2+} ions.

In previous papers, we showed that the frog taste responses to salts, distilled water (Kashiwagura *et al.*, 1977; Kamo *et al.*, 1978) and amino acids (Yoshii *et al.*, 1981) are enhanced by removal of Ca^{2+} ions from the taste receptor membranes. The effect of Ca^{2+} ions on the acid response shown by the present study is opposite to that on the responses to other taste stimuli—the presence of Ca^{2+} ions in an adapting solution leads to enhancement of the acid response. Thus the enhancing effect of Ca^{2+} ions on the taste response is specific to the receptor site for acids. The present results may be interpreted as follows. The responses to acids are induced by binding of protons to the negative receptor sites on the microvillus membrane (Beidler, 1971). The binding site for Ca^{2+} ions is near the receptor site for protons and the binding of Ca^{2+} ions to the site leads to an increase in the number of the receptor sites for protons. Under the normal conditions, the Ca^{2+} ion binding site is not exposed and hence Ca^{2+} ions applied externally to the tongue do not reach the binding site. Changes in conformation of the taste receptor membrane induced by perfusion of the lingual artery or by adaptation of the tongue to a solution of low ionic strength, bring about the exposure of the binding sites, which leads to the binding of Ca^{2+} ions applied externally. Alternatively, the binding of proton on the receptor membrane leads to depolarization of taste cells, which leads to the influx of Ca^{2+} ions from the external solution into taste cells through the microvillus membranes. Further study will be needed to clarify which mechanism actually contributes to the enhancing effect of Ca^{2+} ions on the acid response.

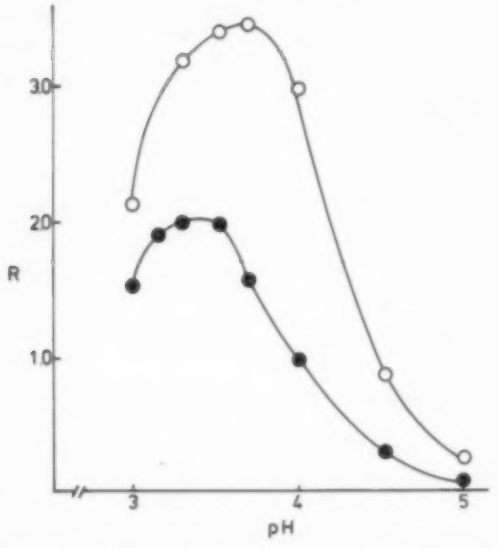

Fig. 3. The magnitude of the response (R) to HCl as a function of pH of the adapting solutions containing 2 mM (●) and 10 mM (○) $CaCl_2$. The lingual artery was perfused with normal Ringer Solution. The magnitude of the response to HCl solution of pH 4 (adapting solution contains 2 mM $CaCl_2$) is taken as unit in the ordinate. Each point in the figure represents the mean value of the data obtained from at least three different preparations.

SUMMARY

The responses of the frog glossopharyngeal nerve to HCl and acetic acid were measured when the lingual artery was perfused with Ringer solution and the effects of allowing the tongue to adapt to solutions of varying concentrations of $CaCl_2$ on the responses to the acids were examined. The results obtained were as follows:

1. The responses to the acids were greatly enhanced by increasing the concentrations of Ca^{2+} ions in a solution to which the tongue had adapted, while the responses to other stimuli such as $CaCl_2$, NaCl, quinine hydrochloride or distilled water were practically independent of the calcium concentration in the adapting solution.

2. An increase in the concentration of Mg^{2+}, Na^+ and K^+ ions in the adapting solution did not lead to any enhancement of the acid responses.

3. An increase in the calcium concentration did not affect the threshold concentration for HCl but led to enhancement of the response at each pH by a factor of about 3.

Two possible explanations of the obtained results are as follows. The binding of Ca^{2+} ions to a receptor site for protons is needed for the binding of protons to the receptor site, or the depolarization of a taste cell induced by the proton binding leads to the influx of Ca^{2+} ions from the external solution into the taste cell.

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ELECTROTONIC COUPLING AND AFTERDISCHARGES IN THE LIGHT GREEN CELLS: A COMPARISON WITH TWO OTHER CEREBRAL GANGLIA NEUROSECRETORY CELL TYPES IN THE POND SNAIL, *LYMNAEA STAGNALIS*

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Abstract—1. Electrotonic coupling occurs between the growth hormone-producing Light Green Cells on the same but not the opposite side of the brain of *Lymnaea*.

2. Long duration inhibitory post synaptic potentials occur spontaneously or can be evoked by nerve or connective stimulation.

3. Afterdischarges, lasting for up to 1 min, are evoked by brief stimulation of the median lip nerves. Activity is confined to cells ipsilateral to the nerve being stimulated.

4. The electrical properties of the Light Green Cells are compared with those of the Caudodorsal Cells and Canopy Cells.

INTRODUCTION

The Light Cells of the pond snail, *Lymnaea stagnalis*, are neurosecretory neurones with a well substantiated role in growth regulation (Geraerts, 1976; Dogterom *et al.*, 1979; Dogterom and Jentjens, 1980; Dogterom and van der Schors, 1980; Dogterom and Doderer, 1981), a function not so far ascribed to any other neurosecretory cell type in gastropod molluscs. Their cell bodies form easily identified clusters on the surfaces of the cerebral ganglia (Joosse, 1964) and their axons project as a bundle to the median lip nerve (Joosse, 1964; Benjamin *et al.*, 1980) which is the neurohaemal organ for the cells. Following our preliminary work on the electrical properties of the Light Green Cells (Benjamin *et al.*, 1976), we report here on the more detailed characteristics of this cell type and compare them with two other types of neurosecretory neurones—the Canopy Cells and Caudodorsal Cells—which also occur in the cerebral ganglia of *Lymnaea*. It will be shown that the Light Green Cells are electrotonically coupled and that brief antidromic stimulation leads to sustained firing (afterdischarges) similar to that which occurs in the egg laying hormone-producing bag cells of *Aplysia* (Kupfermann and Kandel, 1970).

MATERIALS AND METHODS

Standard electrophysiological techniques (Benjamin and Rose, 1979) were used to record intracellularly from up to 4 neurons at the same time in the isolated brain of *Lymnaea stagnalis*. Signals were fed into current injection amplifiers and permanent records made on a penrecorder or filmed from the face of the oscilloscope. Isolated brains were maintained in Hepes (Benjamin, 1978) or phosphate buffered saline (Winlow and Benjamin, 1976). The phosphate buffered saline was also prepared with $\times\frac{1}{2}$ normal calcium and up to $\times 10$ normal magnesium concentration. This was sufficient to block most chemical synapses if brains were bathed for periods of more than 3 hr. Square voltage pulses were applied to the cerebral commissure or median lip nerves (left and right separately) using suction electrodes (Fig. 1).

Figure 1 shows the cell body locations of the three types of neurosecretory neurons investigated. The Light Green Cells and Caudodorsal Cells were bilaterally symmetrical clusters of neurons occurring on the dorsomedial surfaces of the cerebral ganglia (Fig. 1a). There were two clusters of Light Green Cells in each cerebral ganglion (the so-called mediodorsal and laterodorsal cells) and one cluster of Caudodorsal Cells. In total there were about 200 Light Green Cells and 100 Caudodorsal Cells (Joosse, 1964). The Canopy Cells were a pair of individually identifiable neurons whose cell bodies lie in the lateral lobes of the cerebral ganglia (Fig. 1b).

The Caudodorsal Cells and Light Green Cells were located on the surfaces of the cerebral ganglia and could be separated on the basis of their colour, the Caudodorsal Cells usually being paler than the white-orange Light Green Cells. The two cell types were less easy to distinguish at the boundaries of their distributions where the cell bodies lie close together (Fig. 1a). The validity of this colour difference as a means of identification was confirmed in earlier experiments by marking cells with Procion Yellow and staining sections of marked cells with neurosecretory stains using a method described elsewhere (Benjamin *et al.*, 1980). The Light Green Cells took up the stains Alcian Blue and Alcian Yellow in histological sections, whereas the Caudodorsal Cells reacted negatively to these stains but were phloxinophilic (Joosse, 1964).

RESULTS

Spike adaptation and synaptic inputs

Light Green Cells were usually silent but in a few percent of preparations spike activity was seen for a

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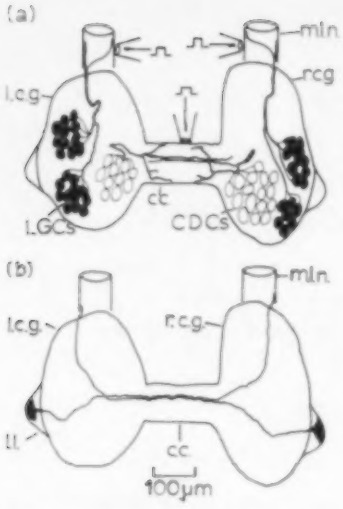

Fig. 1. Cell body locations and axonal projections of the three types of *Lymnaea* neurosecretory neurons discussed in this paper. (a) Diagram of left cerebral ganglion (l.c.g.) and right cerebral ganglion (r.c.g.) from the dorsal view showing the location of cell body clusters of Light Green Cells (LGCs, filled circles) and Caudodorsal Cells (CDCs, open circles). Axons of the Caudodorsal Cells from the left and right cerebral ganglia (total about 100 cells) project to the cerebral commissure (c.c.) which is the neurohaemal organ for these cells. Axons of the two clusters of Light Green Cells in each cerebral ganglia (Mediodorsal Cells, the anterior cluster in each ganglion, and Laterodorsal Cells, the posterior cluster) project to the median lip nerves (m.l.n.) on the ipsilateral side only. These nerves originate from the ventral lobe of the cerebral ganglia (not shown) on each side and are the neurohaemal organs of the Light Green Cells. There are about 200 Light Green Cells in the four clusters. (b) Similar view of the cerebral ganglia showing the location of the cell bodies of the two Canopy Cells in the lateral lobes (l.l.) of the cerebral ganglia. The axons of the cells project contralaterally to the median lip nerves (their neurohaemal organs) of the opposite cerebral ganglia via the cerebral commissure. Suction electrodes were applied to the median lip nerves (see (a)) to provide antidromic stimulation to the ipsilateral Light Green Cells and contralateral Canopy Cells and to the cerebral commissure to provide antidromic stimulation to both left and right clusters of the Caudodorsal Cells.

short period after initial penetration. Figure 2a shows that this spike activity was 1:1 in three randomly selected cells from the same preparation. This spike activity could be modulated by spontaneous inhibitory postsynaptic potentials which made the firing irregular (Fig. 2b). Spiking could be initiated in Light Green Cells by depolarising square current pulses but the cells usually adapted to long duration stimuli. An extreme example of spike adaptation occurred in Fig. 3a where only a single spike could be generated at the beginning of a 1-sec just-suprathreshold depolarising pulse, with none occurring during the subsequent pulses of the same strength. Other Light Green Cells from the same or different preparations showed this spike adaptation to a lesser degree. In Fig. 3b several spikes were produced in response to the first 1-sec depolarising pulse but there was a progressive reduction in the

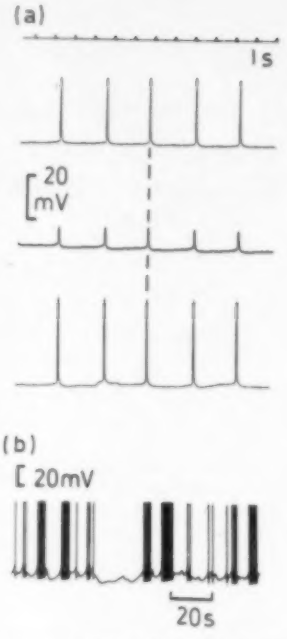

Fig. 2. Spontaneous activity of the Light Green Cells. Spike activity is 1:1 in the three left cerebral ganglion Cells of (a). In (b) activity is modulated by spontaneous inhibitory postsynaptic potential inputs.

number of spikes per stimulus with subsequent pulses of a stimulus train. In a few Light Green Cells the tendency for spike adaptation was not apparent (Fig. 3c). It was interesting that although a particular Light Green Cell often adapted rapidly to a long duration single depolarising pulse (Fig. 3d) the same cell could

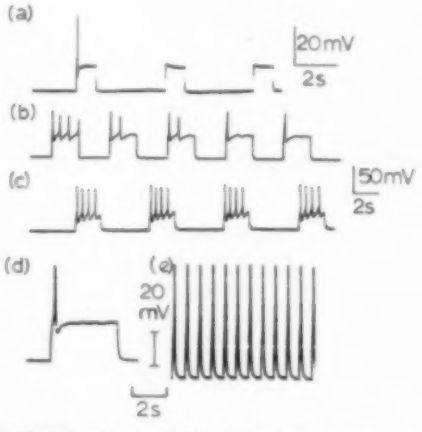

Fig. 3. Variation in the spike adaptation properties of the Light Green Cells. Adaptation of an extreme form is shown in (a) where only a single spike is seen at the beginning of the first depolarising square current pulse of the train. More gradual adaptation to a constant depolarising pulse is shown in (b) whereas in (c) little obvious adaptation occurs. The cell in (d) adapted rapidly to a 1 sec depolarising pulse, but the same cell in (e) fired at high frequency to short duration current pulses (30 msec) of the same amplitude applied at 5 stimuli sec^{-1}.

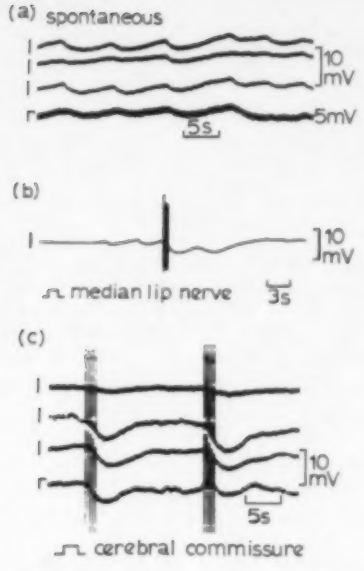

Fig. 4. Long duration inhibitory postsynaptic potentials in the Light Green Cells. (a) Spontaneous inhibitory postsynaptic potentials synchronous in four Light Green Cells from the left (l) and right (r) cerebral ganglia. (b) Inhibitory postsynaptic potentials already present in a left cell are enhanced by a stimulus train applied to the left median lip nerve. This prevented any afterdischarges normally evoked by stimulation of this nerve. (c) Inhibitory postsynaptic potentials evoked in four Light Green Cells by stimulation of the cerebral commissure. In this preparation there were no spontaneous inhibitory postsynaptic potentials present.

be driven at frequencies as high as 5 impulses sec^{-1} by short duration repetitive stimulation (Fig. 3e).

Spontaneous inhibitory postsynaptic potentials of 5–10 sec duration occurred in the Light Green Cells in about 20% of preparations (Fig. 4a). Extracellular stimulation of nerves or connectives evoked similar inhibitory postsynaptic potential inputs when these were absent (Fig. 4c) or increased their amplitude if they were already present (Fig. 4b). Simultaneous recordings from randomly selected Light Green Cells showed that synchronous inhibitory postsynaptic potentials occurred in cells from both the same and opposite cerebral ganglion when inhibitory postsynaptic potentials were present in a particular preparation (Fig. 4a). When they occurred, inhibitory postsynaptic potentials were present in all cells of a randomly selected sample, and their similar waveform and duration (Fig. 4a) suggested a common source of interneuronal input, either a single interneurone or a group of closely coupled cells.

Electrotonic connections between the Light Green Cells

Electrotonic junctions were found between Light Green Cells in the same cerebral ganglion but not between cells in the opposite cerebral ganglia (Fig. 5a) and this reflects the anatomical arrangement of the axons (Benjamin *et al.*, 1980). Thus the axons of the Light Green Cells were confined to the ipsilateral ganglion where they form a bundle projecting to the median lip nerve on the same side (Fig. 1a). Electrotonic coupling was demonstrated by applying square current pulses to one cell and recording similar but attenuated responses in one or more cells of the same type (Figs 5a, 5b, 5c). Responses were obtained from all randomly selected cells so that it appeared that all the ipsilateral Light Green Cells were coupled to each other. These responses persisted when the preparations were bathed for more than 3 hr in high Mg^{2+},

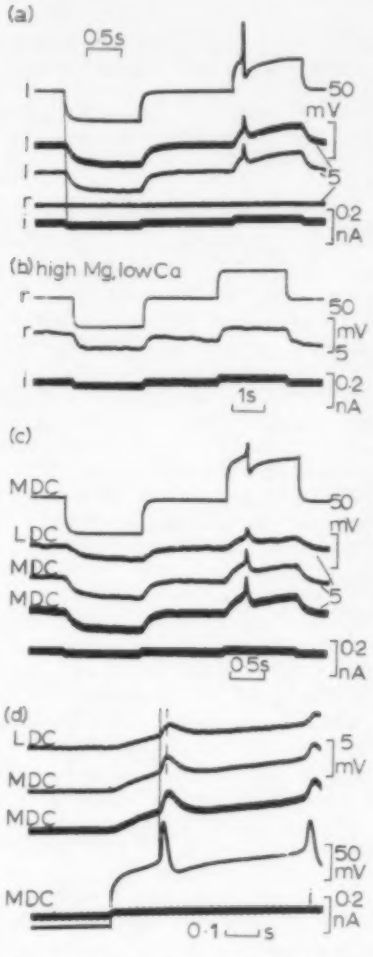

Fig. 5. Electrotonic coupling of the Light Green Cells. Coupling was demonstrated in a–c by passing successive hyperpolarising and depolarising square current pulses of the same current strength into the cell of the top traces (current i, monitored on the bottom trace) and recording similar but attenuated voltage changes in other cells of the same type. (a) Coupling between three cells in the left (l) but no response in the cell from the right (r) cerebral ganglion. (b) Coupling between two right cells after 5 hr in high Mg^{2+}, low Ca^{2+} saline. (c) More distally located cells have a lower coupling ratio than cells close together. Recording is of three mediodorsal cells (MDC) and one laterodorsal cell (LDC, see Fig. 1 for locations). MDC–MDC coupling ratios were both 0.04 whilst the MDC–LDC was 0.03, for both polarities of current injection, indicating non-rectifying junctions. Spikes evoked by depolarising pulse produce biphasic postsynaptic potentials in other cells. (d) Electrotonic postsynaptic potential on a faster time base showing that the time to peak for the depolarising phase of the postsynaptic potential is slightly delayed (dashed line) in the more distally located LDC compared with the MDC.

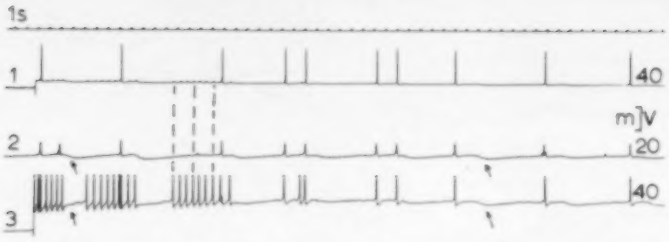

Fig. 6. Electronic coupling and synchronised firing in the Light Green Cells. Steady depolarising current injected into cells 1 and 3. Occasional synchronised firing occurs in all cells at the beginning of the records but only later when cell 3 was firing below 1 spike sec^{-1} did continuous 1:1 firing occur. Dashed lines between the traces indicate that spikes in cell 3 are 1:1 with postsynaptic potentials in cell 1. Arrows show long duration inhibitory postsynaptic potentials which periodically modulate the spike activity of all three cells. Note the doublet spikes in cell 3 which only occur when cell 1 and 2 are also active.

low Ca^{2+} saline (Fig. 5b). No delay could be recorded between the onset of a potential change in one cell and that in other simultaneously recorded cells (shown for spikes and post-synaptic potentials in Fig. 5d and square current pulses in Fig. 5a, solid lines). Electrotonic junctions were non-rectifying, indicated by the fact that the ratio of potential change in the postsynaptic cell to that in the presynaptic cell (coupling ratio) was similar for both depolarising and hyperpolarising square current pulses of the same strength (Fig. 5c). The values of these coupling ratios, in preparations where there were no spontaneous inhibitory postsynaptic potential inputs, were in the range 0.01–0.09 but the strength of coupling was quite variable between different Light Green Cells, even within the same preparation. A general point was that values for coupling ratios were greater for cells within one of the two ipsilateral clusters (the mediodorsal cells and laterodorsal cells, see Fig. 1a) than between clusters (Fig. 5c) and this might be explained by the greater conduction distances involved for the cells in different clusters. Similar spatial factors also accounted for the delayed peak depolarisation of the electrotonic post synaptic potential seen in the top trace of Fig. 5d (dashed lines).

One commonly proposed function for non-rectifying electrotonic junctions is in synchronising the spike activity of coupled cells, and this has been tested in the Light Green Cells by passing steady current into one or more cells and examining the effects on other randomly selected cells of the same type. Figure 6 shows that applying steady current to two or three Light Green Cells eventually led to synchronised firing of all three cells. Cell 3 was particularly strongly activated and to begin with, when cell 3 was firing at its highest frequency, 1:1 spikes only occurred occasionally and postsynaptic potentials occurred in cells 1 and 2 as a result of its spike activity (dashed lines). Later in the record, following adaptation of the cell 3 firing rate, all three cells fired synchronously and this persisted for as long as the cells were depolarised. The activity in all three cells was modulated by inhibitory postsynaptic potential inputs (arrows) and this was an important factor determining the firing pattern, particularly at the beginning of the traces in cell 3 where inhibitory postsynaptic potentials were responsible for the bursts (first arrow).

An important feature of the records in Fig. 6 was the doublet spikes which occurred in cell 3 in the early part of the record whenever spikes were activated in cells 1 and 2. Careful examination of these records showed that the second spike in the cell 3 doublets occurred slightly after the spikes in cells 1 and 2 and were presumably due to reactivation. The fact that Light Green Cells firing asynchronously can reactivate each other is significant because it may play a role in the sustained discharges following antidromic stimulation (Figs 7c and 7e).

Antidromic stimulation and afterdischarges

A short train of antidromic stimuli applied to the Light Green Cells evoked a prolonged after discharge which lasted up to 1 min but more typically between 20 and 30 sec (Fig. 7c). Antidromic stimulation was carried out by extracellular stimulation of the median lip nerves which are known from anatomical studies (summarised in Fig. 1a) to contain Light Green Cell axons. Stimulation of the left and right median lip nerves provided separate stimulation of the ipsilateral Light Green Cells (Fig. 7c). We were confident that the stimulation we applied to the Light Green Cells was antidromic rather than orthodromic because we still got 1:1 spikes following each stimulus pulse after long periods in high Mg^{2+}, low Ca^{2+} saline (Fig. 7a).

The type of discharge seen in Light Green Cells depended on whether the Light Green Cells were already active or not. Stimulation of already active cells caused an acceleration of spike activity which was 1:1 in cells recorded from the same ganglion (Fig. 7b). However, if Light Green Cells were silent—the usual situation in most preparations—then afterdischarges occurred which lasted for 20 sec or more with no obvious 1:1 firing (Fig. 7e) and with a tendency for spike activity to occur in bursts (Fig. 7c). These bursts were most obvious towards the end of the afterdischarge shown in Fig. 7c where bursts tended to be synchronous but not with the same number of spikes per burst in different cells. It is important to note that afterdischarges only occurred in Light Green Cells on the same side as the stimulated nerve and not in cells from the opposite ganglia (Fig. 7c). The responses of the Light Green Cells declined with repeated stimulation but returned to initial response levels after a period of rest of about 1 hr. Not all preparations showed afterdischarges and

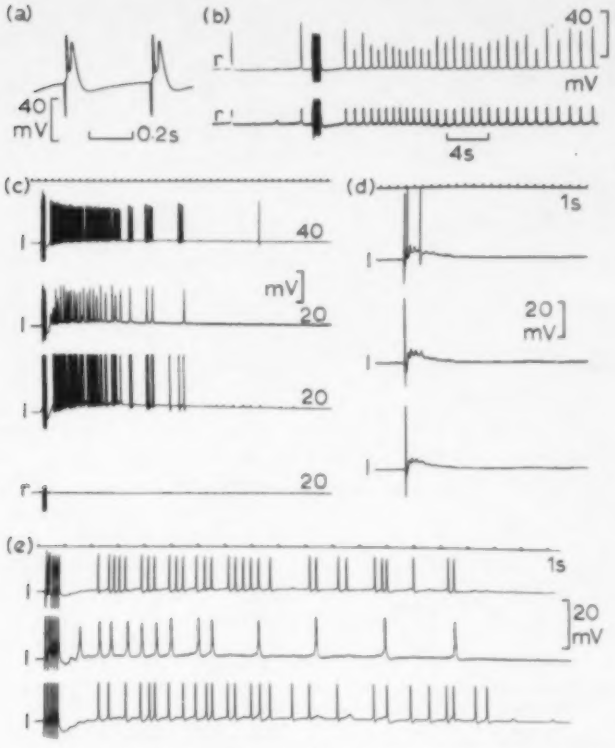

Fig. 7. Antidromic stimulation and afterdischarges in the Light Green Cells. (a) Antidromic spikes in a Light Green Cell produced by median lip nerve stimulation in a preparation which had been bathed in high Mg^{2+}, low Ca^{2+} saline for about 5 hr. The resting potential of this cell is about -80 mV compared with an initial value of about -60 mV in normal saline. To produce antidromic spikes it was necessary to depolarise the cell with intracellular current injection to normal levels of membrane potential. (b) Antidromic stimulation applied to the right median lip nerve in a preparation where the two right (r) cerebral ganglion Light Green Cells were already firing in a 1:1 manner. Antidromic stimulation for a short period results in an increase in firing rate and a persistence of 1:1 firing. (c) Application of stimuli to the left median lip nerve results in afterdischarges in three previously silent cells from the left (l) cerebral ganglion, but nothing in a fourth cell from the right (r) cerebral ganglion. Notice the absence of 1:1 firing and the tendency of the left Light Green Cells to burst. (d) A single stimulus applied to a fatigued preparation produces spikes in the cell of the top trace but not in the other three cells. However, the waveform of the depolarising potential normally underlying afterdischarges can be seen clearly. The hyperpolarising wave immediately following the antidromic spike is followed rapidly by a much longer duration depolarising wave upon which are superimposed electrotonic postsynaptic potentials from other Light Green Cells. The depolarising wave gradually declined after several seconds. (e) Afterdischarges on a faster timebase showing the lack of synchrony of firing in the three left cells. Small potentials appear on the baseline of the recordings, some of which have the appearance of electrotonic postsynaptic potentials. This suggests that feedback from other Light Green Cells might be occurring and some of the summated waveforms gave rise to spikes indicating reverberation. It is impossible to correlate particular postsynaptic potentials with spikes in the recorded cells presumably because many other Light Green Cells are active in the same preparation.

this was particularly the case in preparations where there was a strong spontaneous inhibitory postsynaptic potential input. Stimulation of the median lip nerve here caused an enhancement in the amplitude of the inhibitory post synaptic potential with no afterdischarge (Fig. 4b).

An important feature of the Light Green Cell afterdischarges was the long lasting depolarising wave which underlay the spike activity. A strong hyperpolarising wave initially followed a small train of antidromic spikes (Figs 7c, 7d and 7e). The depolarising wave followed this and gradually declined over the next 20 sec or so (Fig. 7c). This is particular clear in the fatigued preparation of Fig. 7d where the depolarising wave was only of short duration.

The depolarising wave underlying the afterdischarges of the Light Green Cells could be due to some intermediate interneurone(s) whose effects were mediated by chemical synaptic connections. We attempted to test for this by bathing the preparation in high Mg^{2+}, low Ca^{2+} salines but this experiment was inconclusive. Afterdischarges could not be elicited under these circumstances but this could be accounted for by the hyperpolarising effects of the saline on the Light Green Cells rather than the blockage of chemical synapses *per se*. The normal value of membrane potential for the Light Green

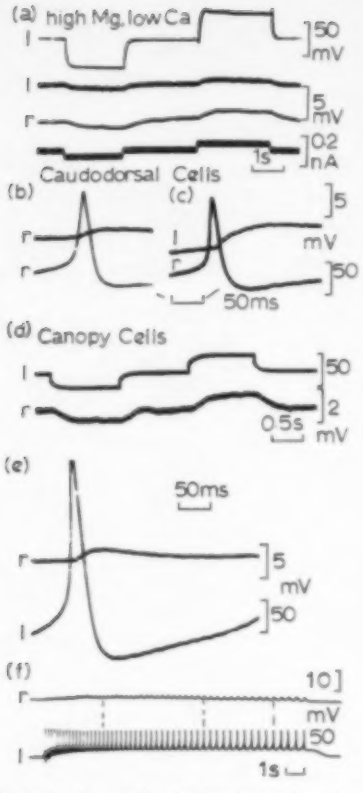

Fig. 8. Electrotonic coupling in the Caudodorsal Cells and Canopy Cells. (a) Successive application of depolarising and hyperpolarising square current pulses into one left (l) Caudodorsal Cell (top trace) produces similar but attenuated voltage changes in another left Caudodorsal Cell and a cell of the same type from the opposite right (r) cerebral ganglion. The preparation had been bathed in high Mg^{2+}, low Ca^{2+} saline for 3 hr. Current (i) monitored in the bottom trace. (b) Pair of Caudodorsal Cells cells recorded in the same cerebral ganglia to be compared with a pair recorded from the opposite cerebral ganglion (c). In (c) a spike in one cell evokes an electrotonic postsynaptic potential whose onset depolarisation is delayed compared with (b). (d) Non-rectifying electrotonic coupling of the Canopy Cells. Depolarising and hyperpolarising currents of same strength injected into the left Canopy Cell. Coupling ratio is 0.03 for both current polarities. (e) Electrotonic postsynaptic potentials with delay in the right Canopy Cell following a spike in the left Canopy Cell. (f) Spikes evoked in the left Canopy Cell produce 1:1 electrotonic postsynaptic potentials in the right Canopy Cell but no synchronised spikes.

Cells was between −55 and −60 mV but in high Mg^{2+}, low Ca^{2+}saline this increased to about −80 mV. This not only made it difficult to evoke cell body spikes by antidromic stimulation (see legend for Fig. 7a) but also prevented afterdischarges by raising the threshold for spike initiation in all the Light Green Cells.

In other electrotonically coupled networks, such as the pleural ganglia of *Tritonia* (Getting and Willows, 1974), positive feedback within the network resulting from asynchronous spike activity led to a gradual build up of activity within the network. This is also a possible mechanism for sustaining activity in the Light Green Cells because asynchronous activity occurs in their afterdischarges (Fig. 7e). We have already noticed that this can lead to reactivation in situations where the cells are activated by intracellular stimulation (see Fig. 6a) but it was difficult to obtain conclusive evidence that this occurred during afterdischarges because of the large numbers of cells simultaneously active (about 100 cells in each cerebral ganglion). Thus in the afterdischarges shown in Figs 7c and 7e small, probably electrotonic, postsynaptic potentials can be seen interspersed between spikes, but their complex waveforms made it difficult to correlate postsynaptic potentials in one cell with spike activity in other recorded cells, thus making it impossible to provide detailed evidence for positive feedback.

Caudodorsal Cells and Canopy Cells

Comparing the electrical properties of the Light Green Cells with two other cerebral ganglia neurosecretory cell types showed that electrotonic coupling was a common feature of neurosecretory cells in *Lymnaea*. Low d.c. coupling ratios of about 0.03 were obtained for the Canopy Cells (Fig. 8d) and action potentials in one cell were incapable of producing synchronous spike activity in the other (Fig. 8f). Unlike the Light Green Cells where coupling was confined to one ganglion, those between Caudodorsal Cells occurred between cells on both the same and opposite sides of the brain (Fig. 8a). Delays between the onsets of spikes and postsynaptic potentials occurred between cells on the same side (Fig. 8b) and this was presumably due to conduction delays. A similar delay occurred in the case of the Canopy Cells (Fig. 8e) which were also located on the opposite side of the brain (Fig. 1b).

In no preparation were we able to elicit afterdischarges by antidromic stimulation of the Canopy Cells, but these were obtained in the case of the Caudodorsal Cells (Fig. 9). Previous work by de Vlieger *et al.* (1980) showed that the Caudodorsal Cells (like the homologous functional cell type in *Aplysia*—the bag cells—Kupfermann and Kandel, 1970) were capable of carrying out prolonged afterdischarges lasting up to 1 hr following intracellular activation of single cells. In our experiments we antidromically stimulated the Caudodorsal Cells by suction electrodes placed on the cerebral commissure (Fig. 1a) and obtained much shorter afterdischarges (Fig. 9) often lasting from 6–10 sec but sometimes up to 1 min. The interesting feature of these afterdischarges was that firing in both left and right Caudodorsal Cells was 1:1 from the start (Fig. 9) and so positive feedback between cells was not necessary for sustained activity to occur. Furthermore, each spike was followed by a depolarising afterpotential (Fig. 9, arrows) which contributed to the depolarising wave underlying the afterdischarge. Similar afterdepolarisations have been implicated in the formation of bursts in some molluscan endogenous bursters (Thompson and Smith, 1976; Bulloch and Willows, 1981) and could also be involved in part of the sustaining mechanism in the Caudodorsal Cells. A final point was that the Caudodorsal Cells' afterdischarges were terminated suddenly by a mechanism which produced a shoulder at the end of the depolar-

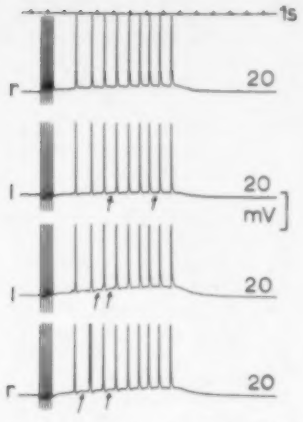

Fig. 9. Afterdischarges in the Caudodorsal Cells. Application of a train of stimuli to the cerebral commissure resulted in 1:1 spike afterdischarges in two Caudodorsal Cells from the right (r) cerebral ganglion and two from the left cerebral ganglion (l). Underlying these discharges is a long depolarising wave which starts after the last antidromic spike and terminates suddenly to form a shoulder after the last spike in the afterdischarge. Afterdepolarisations (arrowed) from about 0.5–1 sec in duration follow each spike in the afterdischarge.

ising wave rather than the gradual fall in depolarisation seen in the Light Green Cells (Figs 7c and 7d).

DISCUSSION

Electronic coupling and synchronised firing

Evidence presented in this paper has shown that the electrotonic coupling of *Lymnaea* neurosecretory neurones was capable of producing 1:1 firing under certain conditions. It occurred in the Light Green Cells, but spikes in one cell of the network did not inevitably give rise to synchronised spikes in all cells of the same type. This could be due to differences in threshold and excitability of different cells but was also a function of the frequency of firing of the cells, in that consistent 1:1 firing only occurred at a frequency below about 1 sec^{-1}. Synchronised firing of Caudodorsal Cells was more readily obtained by antidromic stimulation than by intracellular injection of current and in this case firing was 1:1 in cells from both left and right cerebral ganglia. No synchronised firing was seen in the Canopy Cells by either method of activation although previous work had shown that common inhibitory inputs could cause similar burst patterns of activity, but not with close synchrony of individual spikes (Benjamin *et al.*, 1976).

Spontaneous 1:1 firing was sometimes seen in the Light Green Cells and we presume that it was also mediated by electrotonic coupling. The mechanism responsible for this activation was uncertain although it could be due to general depolarisation caused by electrode impalement of a number of cells over a short period of time. It tended to occur in the initial part of an experiment and die away with time.

Afterdischarges

The Light Green Cells are the second type of molluscan neurosecretory neurone capable of showing afterdischarges. The first type to be discovered were the bag cells of *Aplysia* (Kupfermann and Kandel, 1970) and later the Caudodorsal Cells of *Lymnaea* (de Vlieger *et al.*, 1980) both of which produce an ovulation-inducing hormone (e.g. Arch, 1976; Geraerts and Bohlken, 1976). In these cells the massed and sustained electrical discharge was presumed to mediate the phasic release of hormone necessary for egg release and associated behaviour to occur. In the case of the growth hormone producing (Geraerts, 1976) Light Green Cells nothing is known about how the hormone is normally released and what inputs are normally responsible for their electrical activation. In isolated brains the cells are normally silent and need some further activating input to cause release of their hormone into the blood. No clue to this input was revealed by nerve or connective stimulation because this evoked or enhanced the amplitude of long duration inhibitory postsynaptic potentials which actually prevented afterdischarges being initiated.

The afterdischarges evoked in the Caudodorsal Cells in the present experiments could last for 1 min or more but often ceased after 6–10 sec. This was shorter than the afterdischarges seen in the bag cells of *Aplysia* (Kupfermann and Kandel, 1970) and this can partly be explained by the different methods of stimulation used in the two preparations. Asymmetrical activation of bag cells on one side of the abdominal ganglion led to a prolongation of activity in the bag cells because of reverberation between left and right populations (Kupfermann and Kandel, 1970). Cerebral commissure stimulation in *Lymnaea* activated both left and right Caudodorsal Cell populations at the same time and firing patterns were 1:1 on both sides. This lack of reverberation between Caudodorsal Cells within the same and opposite ganglia (Fig. 9) suggested that the electrotonic coupling of the Caudodorsal Cells, in the circumstances of the present experiments, was not the most important factor causing the afterdischarges, although it could be important in synchronising activity once they were activated. In fact we were unable to activate the Caudodorsal Cells by intracellular injection of current into individual cells and this suggests that our preparations were basically less easily activated than those used by de Vlieger *et al.* (1980), where intracellular activation of only one cell could cause sustained discharges lasting up to 1 hr (although this was highly variable). A likely explanation for this was that we used snails for autumn and winter months whereas the Dutch groups used non-seasonal animals which were in continuous egg laying condition. A seasonal effect may have made our snails less susceptible to activating stimuli although we did keep some snails on a 12:12 LD cycle, which tended to bring them into reproductive condition, with no obvious effect.

The Light Green Cells were similar to the Caudodorsal Cells in that they showed afterdischarges lasting for up to 1 min. However, they differed from Caudodorsal Cells in having electrotonic junctions confined to cells from the same ganglion, so that reverbatory activity between left and right cerebral ganglion populations was not possible. Asyn-

chronous firing of Light Green Cells did occur in cells from the same ganglion and here reverberation could be playing a role in generating the afterdischarge, but even amongst ipsilateral cells synchronous increases in firing rate did occur if the cells were already active, so in some circumstances both the Caudorsal Cells and Light Green Cells were both capable of sustained discharges in the absence of reverberation.

We suggest that an important factor in the afterdischarges of the Caudodorsal Cells were the depolarising afterpotentials which followed each spike in the afterdischarge. These appeared to be intrinsically generated potentials consequent on spike initiation. Similar afterdepolarisations have been observed in R15 of *Aplysia* (Thompson and Smith, 1976) and the Yellow Cells (Benjamin, 1978) and Light Yellow Cells of *Lymnaea* (van Swigchen, 1979). They were thought to contribute to the sustained discharge in the endogenous bursts of these neurons and may play a similar role in the Caudodorsal Cells. In R15 a second mechanism came into play after a few seconds which hyperpolarised the cell and was responsible for the initial stages of the interburst interval (Junge and Stephens, 1973). Such a mechanism, if it occurs in the Caudodorsal Cells of *Lymnaea* where a delayed increase in membrane potential also occurred (the shoulder in Fig. 9), must be very slow in its development compared with endogenous bursters such as R15.

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FIBRE COMPOSITION AND ENZYME ACTIVITIES IN FIVE DIFFERENT MUSCLES FROM THE SVALBARD REINDEER

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Abstract—1. βR fibres (type I) constitute less than 10% of the semimembranosus and longissimus dorsi muscles and about twice as much of the gluteobiceps and flexor hallucis.

2. Except for longissimus dorsi, 50% or more consist of αW (type IIB) fibres—in semimembranous, as much as 70%.

3. Despite the comparatively large content of αW fibres, both the oxidative capacity and the capacity to metabolize fatty acids is high.

4. Furthermore, unexpectedly small differences in oxidative capacity between the three fibre types βR, αR and αW (I, IIA and IIB) are revealed by histochemical staining.

5. These results indicate a tendency to bring the three fibre types closer together as regards metabolic activities, as an adaptation to the relatively tranquil life of this animal.

6. However, the large content of αW fibres does not accord well with this way of life, as they guarantee quick movements.

7. The comparatively high oxidative capacity of the αW fibres in the Svalbard reindeer and the fact that during starvation it is primarily αW fibres that contribute to the energy supply by protein degradation may nevertheless account for their abundant occurrence.

INTRODUCTION

Survival mechanisms in animals living under extreme climate and nutritional conditions can assume many forms. The Svalbard reindeer (*Rangifer tarandus platyrhynchus*), which lives in an extremely cold climate during most of the year, is short-legged and square-built and has a very long and thick pelt. Furthermore it has an extreme capacity to lay up fat stores during the summer (Øritsland, 1970, 1980; Reimers and Ringberg, 1983). According to Ringberg (1979) its high growth hormone level during the winter time indicates a high lipolytic activity, that is, a high capacity to utilize fat as substrate when access to feed is short and temperatures are low.

As regards skeletal muscle properties in general there are, put very simply, two main demands to ensure survival, fleetness of foot so as to escape predators, and endurance capacity in the search for food.

We have attempted to explain the Svalbard reindeer's muscle functions in the light of the extreme conditions to which this animal must adapt in order to survive. The present work deals with identification of fibre types and estimation of certain vital metabolic properties in five muscles of the Svalbard reindeer.

MATERIALS AND METHODS

Animals

Animals were killed (by shooting) at the end of June and in early July. The total material, collected on Kap Linné, Nordenskiøld land, consisted of eight adult females and four adult males.

Muscles

Samples were taken from the following muscles: cranial part of M. gluteobiceps, exterior part of M. flexor hallucis long., M. semitendinosus, M. semimembranosus, M. longissimus dorsi.

Histochemistry

For histochemistry, muscle specimens were taken as surgical biopsies, trimmed, oriented, mounted in Cryoform, frozen in isopentane cooled by liquid nitrogen, and stored at −80°C until analysed. Transverse sections (10 μm) were cut with a cryotome and stained for myofibrillar ATP-ase after preincubation at pH 4.3–4.6 (Padykula and Herman, 1955; Guth and Samaha, 1969; Brooke and Kaiser, 1970) and for NADH-dehydrogenase (Novikoff *et al.*, 1961). Depending on the staining technique, the fibres were classified as I, IIA and IIB or βR, αR and αW.

Enzyme activities

Three enzymes were chosen to represent the important pathways in energy metabolism: the respiratory chain by cytochrome oxidase (cytox; EC 1.9.3.1.) fatty acid β-oxidation by 3-hydroxyacyl-CoA dehydrogenase (HAD; EC 1.1.1.35.) and lactate fermentation by lactate dehydrogenase (LDH; EC 1.1.1.27.). Cytox activity was estimated according to Whereat *et al.* (1969), HAD and LDH by the methods of Bass *et al.* (1969). For enzyme activity determinations, biopsy samples (25–50 mg) were homogenized with 19-fold amounts (w/v) of ice-cold potassium bicarbonate, 62 mM, pH 7.4, containing 0.15 M KCl and 6 mM EDTA in a small, ice-cooled, all-glass Potter–Elvehjem homogenizer. The resulting crude homogenate was kept ice-cold and was appropriately diluted prior to activity determinations. The biopsy samples were homogenized either as soon as possible after slaughter (1–2 hr) without previous freeze–thawing, or at the laboratory in Uppsala, in which case the biopsies had been kept frozen in

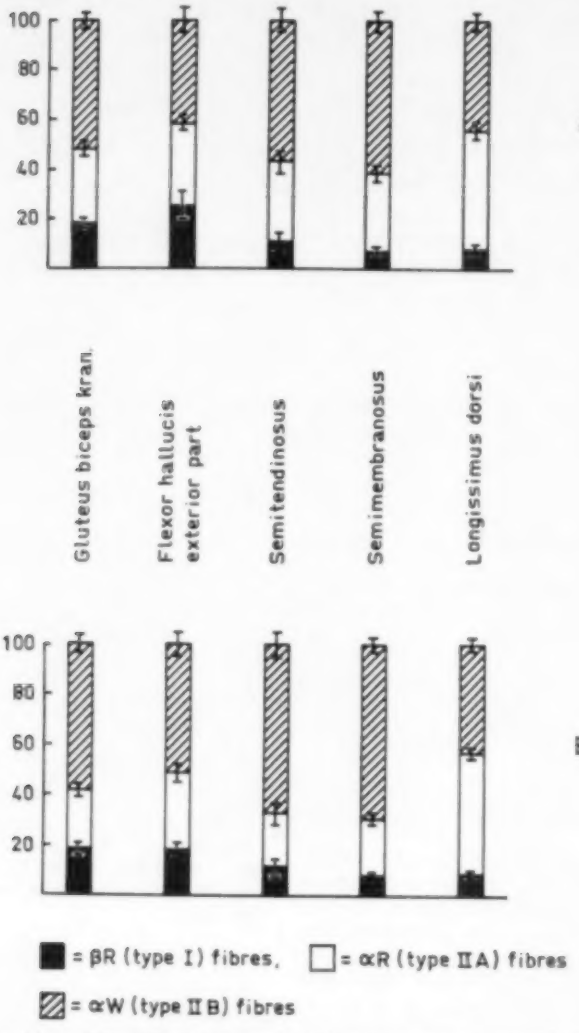

Fig. 1. Fibre composition of five different Svalbard reindeer muscles. The proportion of each fibre type is expressed as relative area, i.e. the area as a percentage, that they occupy in transverse section. The columns are mean values from 9 to 11 animals ± SE. (A) After staining for NADH-dehydrogenase activity. (B) After staining for myosin ATPase activity.

liquid nitrogen during transport. On the first occasion only cytox was estimated; on the second occasion all three enzymes (cytox, HAD and LDH) were measured.

Choice of methods

Two slightly different staining techniques have been used. The first is based mainly on the NADH-dehydrogenase activity, and is a measure of the oxidative capacity of the various fibres. The other is based on the myofibrillar ATPase activity and is a measure of the contractile properties. In the first case the fibres are usually designated βR, αR and αW, where βR stands for β-red and is high oxidative–low glycolytic, αR stands for α-red and is high oxidative–high glycolytic, and αW stands for α-white, which is low oxidative–high glycolytic. In the second case the fibres are designated I, IIA and IIB, where I contracts slowly and IIA and IIB contract rapidly. In previous studies on rat muscle the two techniques showed considerable discrepancies (unpublished results). In the present study, however, they show good agreement, that is, βR, αR and αW correspond fairly well to I, IIA and IIB respectively. A fourth type of fibre, IIC, has not been analysed.

One very important item of information about muscle is its capacity to perform aerobic metabolism, that is, to oxidize substrates efficiently. Cytochrome oxidase activity is a measure of this capacity and has the advantage of being relatively easy to assay. Its great disadvantage, however, is its loss of activity after freeze–thawing of the muscle. The most convenient technique is therefore to measure the enzyme activity in fresh samples. Unfortunately this is not always feasible, especially when samples are collected under field conditions. In the present study, however, we had the advantage of being able to measure cytochrome oxidase capacity within a few hours after the animal was shot. The instrument was certainly primitive but reliable enough to allow an estimation of activity losses by freeze–thawing of the muscle samples. This revealed a 50% less cytochrome oxidase activity after freeze–thawing the material compared with that in fresh material. This is in agreement with results obtained with human muscle by Bylund-Fellenius *et al.* (1982). This has to be kept in mind when we present figures

about cytox activity obtained after freeze–thawing. Of necessity, tissue samples taken in the field far from the laboratory must be transported in liquid nitrogen and, consequently, most studies on animals outside the laboratory are done on previously frozen material. HAD and LDH are only very slightly influenced by freeze–thawing, or not at all.

RESULTS

Fibre composition

Figure 1 shows the fibre composition of the five muscles (A) after staining for NADH-dehydrogenase activity, and (B) after staining for myosin ATPase following preincubation at pH 4.6, as described earlier. No dramatic difference can be seen between the two techniques. βR (I) constitutes less than 10% of the semimembranosus and longissimus dorsi muscles and about twice as much of the gluteobiceps and flexor hallucis. Except for longissimus dorsi, 50% or more of the muscles consist of αW (IIB) fibres; in semimembranosus, as much as 70% (Fig. 1B).

It proved difficult almost throughout to distinguish the three fibre types from each other in the Svalbard reindeer muscles by means of NADH-dehydrogenase staining. Unlike in many other species, the αW (IIB) fibres stained comparatively dark, sometimes even darker than the βR (I) fibres.

Figure 2 shows the activities of the three enzymes cytox, HAD and LDH. No dramatic differences can be seen between the muscles, where longissimus dorsi has the highest and flexor hallucis the lowest oxidative (cytox) capacity. High oxidative capacity is also associated with high β-oxidation capacity of fatty acids (HAD). No direct or inverse relation between these two enzyme activities and lactate dehydrogenase (LDH) activity can be seen.

DISCUSSION

From Fig. 1 it is obvious that whichever of the two methods described above is used to identify the fibres, the results are roughly the same. In four of the five muscles studied, αW fibres (IIB) predominate, and in one, longissimus dorsi, αR (IIA) is predominant. Normally a high αW fibre content means a high glycolytic and low oxidative metabolism. But in the Svalbard reindeer muscles the cytox activity, which has been used as a measure of the capacity to perform oxidative metabolism, is high compared with other ruminants, both wild and domestic (unpublished results) in spite of the comparatively high content of αW fibres. The same is true of HAD, showing a high capacity to metabolize fatty acids (Fig. 2).

NADH-dehydrogenase staining of the muscle fibres is a histochemical measure of the oxidative activity—the darker the fibre, the higher the activity. With this method only minor differences in blackness between αR and αW fibres was found. This indicates very small differences as regards oxidative capacity between these two fibre types which, however, can still be easily distinguished from each other by the myofibrillar ATPase technique (then called IIA and IIB fibres). It is even more confusing that the αW fibres in the Svalbard reindeer sometimes stain even darker than βR fibres with the NADH-dehydrogenase technique. βR fibres are mostly looked upon as high oxidative *vis-á-vis* αW fibres.

Comparison of enzyme activities, shown in Fig. 2, with fibre compositions (Fig. 1) does not reveal any simple connection. Longissimus dorsi, with a very low proportion of β fibres but with the highest contribution of αR fibres in all muscles studied, also shows the highest cytox and HAD activities. Gluteobiceps on the other hand, with a comparatively high proportion of βR fibres but a low share of αR fibres, has only slightly less cytox and HAD activity (20%). Another example is the αW fibre content in semimembranosus, which is 50% greater than in longissimus dorsi, whereas cytox activity is about the same in the two muscles.

These observations indicate a tendency to bring the three fibre types closer together as regards metabolic activities. An explanation for this levelling out of the metabolism of the three fibres may be the comparatively tranquil life of the Svalbard reindeer. As pointed out by Reimers (1980) the absence of harassment from predators, insects and man allows the Svalbard reindeer to maximize its food intake and minimize its energy expenditure, compared with the

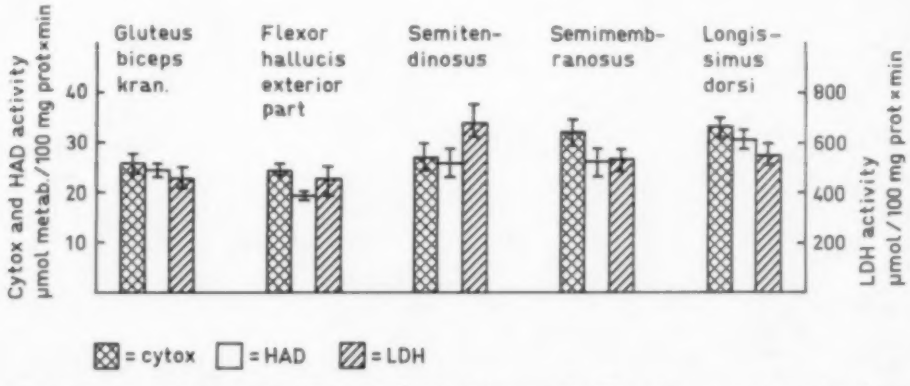

Fig. 2. Enzyme activities in five different Svalbard reindeer muscles. The columns are mean values from 9 to 11 animals ± SE.

mainland reindeer. This makes it possible for the Svalbard reindeer to build up large fat depots during the three summer months with 24 hr daylight and which satisfy a substantial part of their energy requirements during the winter. Metabolic depression in winter further reduces their energy requirement during this season (Øritsland, 1980). On no occasion is there any need for rapid movement as traditionally attributed to muscle fibres specialized in high anaerobic glycolysis. Muscle metabolism for survival should therefore be primarily adapted to a complete oxidative metabolism of endogenous fat, which would explain the high HAD activity compared with other ruminants and relative to oxidative capacity (unpublished results).

Lean tissue is also reduced during winter. According to Reimers and Ringberg (1983), females may lose about 30% of their autumn lean body weight and males even more during the winter. Goldspink and Ward (1979), working with rodent muscle, observed that starvation caused a decrease in size of type II fibres, predominantly type IIB. No change in the number of muscle fibres was found in their experiments and the effect was completely reversible. This protein degradation may contribute considerably to the animal's energy supply and may be one explanatory factor for the comparatively low content of type I fibres in the Svalbard reindeer muscle, in favour of type IIB fibres especially. One might otherwise have expected a high proportion of type I fibres in this animal, in view of the fact that its way of life calls almost exclusively for slow movements.

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HOMEOSTATIC RESPONSES TO WATER DEPRIVATION OR HEMORRHAGE IN LACTATING AND NON-LACTATING BEDOUIN GOATS

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Abstract—1. Three lactating and three non-lactating black Bedouin goats were subjected to four days of water deprivation or to hemorrhage.

2. Four days of water deprivation caused body wt losses of 32 and 23% and plasma volume losses of 30 and 34% in lactating and non-lactating goats respectively.

3. Plasma osmolality increased 17 and 15% in lactating and non-lactating goats. Plasma arginine vasopressin concentration rose from about 5 pg/ml to a mean of 36 pg/ml. Plasma renin activity increased from about 0.7 ng/ml/hr to a mean of 3.45 ng/ml/hr in lactating and to 3.15 ng/ml/hr in non-lactating goats.

4. At 4.5 hr post-rehydration plasma osmolality and plasma vasopressin concentration were back to normal in non-lactating, but still elevated in lactating goats. Plasma renin activity increased after rehydration.

5. Rapid blood volume loss of 21–28% increased plasma vasopressin concentration to 16–35 pg/ml in non-lactating and to 70 or > 500 pg/ml in lactating goats.

6. It is concluded that black Bedouin goats are well adapted to endure severe dehydration and rapid rehydration, but that they (especially lactating animals) react strongly to rapid volume depletion.

INTRODUCTION

Animals inhabiting the desert are expected to be adapted to water shortage. The black Bedouin goats (*Capra hircus*) graze all day fully exposed to sun and dry air which causes large water losses. Yet these goats can endure 4 days without drinking water and still continue to eat and to produce milk. The Bedouin goats are apparently equipped with special characteristics such as high total body water and large plasma volume, which increase even further during lactation (Shkolnik *et al.*, 1972; Shkolnik *et al.*, 1979; Maltz and Shkolnik, 1980; Shkolnik *et al.*, 1980). After 4 days of dehydration the plasma volume is at a level which is considered normal for water replete goats of other breeds (Hix *et al.*, 1959). Moreover, when given access to water, Bedouin goats replenish their water deficit within a few minutes. They can drink up to 40% of their dehydrated body wt without apparent ill effect; this is due largely to the ability of the rumen to prevent the water from entering the blood too rapidly (Choshniak and Shkolnik, 1977a; Choshniak and Shkolnik, 1977b; Shkolnik *et al.*, 1979).

Two hormonal systems are of special importance in the regulation of water balance and maintenance of plasma volume, vasopressin and the renin–angiotensin–aldosterone system. Vasopressin release is regulated principally by the osmotic (Na-) concentration of the extracellular fluid (Verney, 1947; Robertson *et al.*, 1976; Andersson *et al.*, 1980), but its release may be modified by changes in plasma volume. Thus, the sensitivity of cerebral receptors to osmotic stimulation increases during moderate hypovolemia in humans (Robertson and Athar, 1976), dogs (Kozlowsky and Szczepánska-Sadowska, 1975) and rats (Dunn *et al.*, 1973). This sensitization may be mediated by the renin–angiotensin system (Fitzsimons, 1972) and a striking interaction between cerebral Na-sensitive receptors and angiotensin II has been observed (Andersson, 1977). In addition, reflex modulation of the vasopressin secretion by an inhibitory tonus from distension receptors in the heart and pulmonary veins contributes to the homeostatic control of the extracellular fluid volume (Gauer *et al.*, 1970). It seemed of interest to study the plasma vasopressin (plasma arginine vasopressin = pAVP) conc. and the plasma renin activity (PRA) during 4 days of water deprivation in Bedouin goats, which repeatedly have to endure such severe dehydrations. We also compared the level of pAVP during dehydration with that which is seen during rapid hemorrhagic volume depletion.

MATERIALS AND METHODS

Animals

Black Bedouin goats were purchased from Bedouins in the eastern Sinai along the Gulf of Eilat and kept and bred in the research zoo of Tel Aviv University. All goats were penned together outdoors and exposed to solar radiation with no access to shade. (Noon temperature in the shade was 25 ± 2°C and RH 68 ± 5%). Three lactating (3 months post-partum) and three non-lactating goats were used. The body wt of the lactating goats was 25.2 ± 3.1 kg and of the non-lactating ones 24.3 ± 2.5 kg. The animals were routinely kept on low quality hay with resultant low milk yield. A fortnight before onset of the experiments, the diet was changed to alfalfa hay without added concentrates and this diet was maintained throughout

the experimental period. The animals were fed in the morning and in the afternoon. Immediately before afternoon feeding, the animals were given water and allowed to drink to satisfaction. The water was then removed and the kids were allowed to suck. The kids also sucked at 08.00 a.m. The milk yield (17–35 g/kg/day) was calculated from the weight gains of the kids as measured before and after sucking by an electronic balance. At other times the kids were kept separated from their mothers but could be seen and heard by them.

Dehydration experiments

The animals were allowed to drink as usual in the afternoon on day 0 and were then given no water until the evening 4 days later. On the morning of the first day the animals were fed as usual and the kids allowed to suck. The animals were then weighed and a polyethylene catheter inserted into the jugular vein. Blood samples were taken in the morning and afternoon each day. In addition, blood samples for determination of plasma volume were taken morning and afternoon on day 1 and every afternoon on days 2, 3 and 4. After a control sample, Evans blue was injected intravenously and thereafter timed blood samples were taken. After 4 days of water deprivation the animals were given a bucket full of water at 18.00 p.m. and allowed to drink to satisfaction and water consumed was measured. A blood sample was taken within 2 min after drinking, followed by a second sample after 60–90 min and a final sample after 4–4.5 hr.

Haemorrhage experiments

In the morning just before the sun reached the animals, one of the goats was brought indoors, weighed and placed in a metabolic cage in which only the turn around movement was restricted. The temperature of the room varied from 20°C in the morning to 30°C in the afternoon. A polyethylene catheter was inserted into one of the jugular veins and plasma volume (pV) and hematocrit were measured and blood trapping between the red blood cells. At 10.00–11.00 a.m. a second polyethylene cannula was placed in the contralateral jugular vein and the animal was bled at a rate of 20–40 ml/min. The blood was collected in heparinized polyethylene bags. The animals were bled until forced breathing indicated that the arterial blood pressure had fallen, which previously had been shown to coincide with a conspicuous pAVP release (Larsson *et al.*, 1978). The hemorrhage was then stopped. Blood samples for pAVP analyses were taken before, and at regular intervals during and after bleeding. A second measurement of plasma volume was performed at about 2.5 hr post-bleeding in five of the animals. In the sixth animal the hematocrit had fallen as low as 13% and therefore, the blood was immediately returned intravenously. In the other five animals, the blood was returned about 4 hr after the end of bleeding. After the blood had been returned, water was offered.

Analyses

Blood for hematocrit, plasma osmolality (pOsm), Na (pNa), K (pK) and measurements of Evans blue colour, was drawn into heparinized plastic tubes. The hematocrits were obtained by centrifuging (in triplicates) microhematocrit tubes at 4000 rpm in a clinical centrifuge for 20 min. The pOsm was analysed by freezing-point depression (Adv. Instrument osmometer) and pNa and pK by internal standard flame photometry (IL 343). Blood for analysis of pAVP and PRA was taken into pre-chilled tubes containing Na_2-EDTA as anticoagulant. Samples were spun at +4°C and the plasma transferred to plastic tubes (the ones for AVP analyses containing 0.1 ml acetic acid per 1 ml of plasma) which were kept at −17°C until analysed. Plasma AVP was analysed by radio-immunoassay technique (Husain *et al.*, 1973). PRA was analysed by radio-immunoassay as described by Fyhrquist *et al.* (1976).

Plasma volume was determined as the dilution volume of Evans blue (T_{1824}) in the manner described by Maltz and Shkolnik (1980).

The data are presented as mean ± SE of the mean.

RESULTS

Water deprivation

During 4 days of water deprivation the goats lost large volumes of water. Non-lactating goats lost 5.6 ± 2 kg or 23% of their initial body wt (Table 1). The lactating animals lost 7.9 ± 3.1 kg or 32% of their body wt, part of which was due to water loss via the milk. The milk yield was 470 ± 100 ml/day and was maintained at this level during the first two days of dehydration, but decreased to about 35% of the initial yield on the fourth day of dehydration. On the morning of day 1 the lactating goats had plasma volumes similar to those of the non-lactating animals (Table 1). However, as dehydration proceeded the lactating goats retained more fluid in the plasma than the non-lactating animals. On the second day of dehydration the lactating goats had lost 177 ± 63 ml and the non-lactating animals as much as 315 ± 55 ml. At the end of the fourth day the plasma volume had dropped 406 ± 67 ml in lactating goats and 455 ± 16 ml in the non-lactating ones. Thus lactating animals maintained their plasma volume/body wt relationship in contrast to the non-lactating goats (Table 1).

The water loss was reflected in a marked rise in pOsm which was already evident in the evening of day 1 (Fig. 1). The pNa rose from 141 ± 1 to 162 ± 2 mM/l during the four days of water deprivation (lactating goats). In non-lactating animals pNa increased from 146 ± 0 to 164 ± 1 mM/l. Plasma K concentrations showed no significant change in lactating goats (4.4 ± 2 before, and 4.3 ± 0.2 mM/l at the end of water deprivation), but fell slightly in the non-lactating animals (from 4.5 ± 0.2 to 4.0 ± 0.2 mM/l).

Although pOsm had increased markedly the first day of dehydration the increase of pAVP was delayed until the second day (Fig. 1). At the end of the water

Table 1. Changes in body wt and plasma volume during 4 days of water deprivation in three lactating and three non-lactating goats. Mean and SE

	Loss in body wt (% of initial)		Loss in plasma volume (% of initial)		Plasma volume as % of body wt	
	Non-lactating kg	Lactating kg	Non-lactating	Lactating	Non-lactating	Lactating
Initial	24.3 ± 2.5	25.2 ± 3.1	1370 ± 84	1380 ± 135	5.6 ± 0.2	5.5 ± 0.1
Day 2	14 ± 1	19 ± 1	23 ± 4	13 ± 5	5.0 ± 0.1	5.9 ± 0.5
Day 3	20 ± 1	26 ± 1	29 ± 5	26 ± 4	5.0 ± 0.6	5.6 ± 0.3
Day 4	23 ± 1	32 ± 1	34 ± 2	30 ± 4	4.8 ± 0.2	5.6 ± 0.4

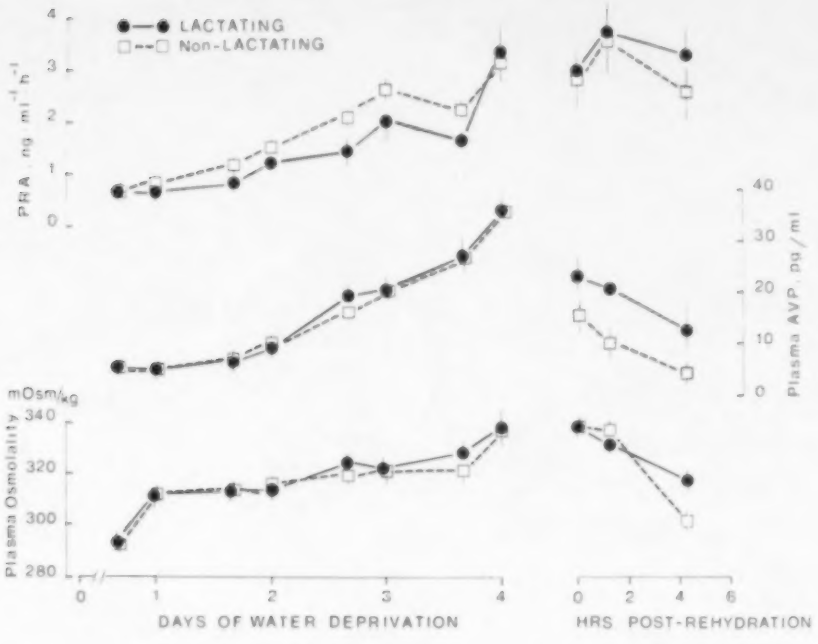

Fig. 1. Plasma renin activity (PRA), plasma osmolality and plasma arginine vasopressin (AVP) concentration during 4 days of water deprivation and 4.5 hr post-rehydration. Lactating goats = 3; non-lactating goats = 3.

deprivation period the absolute increase in pAVP was similar in both groups.

On day 2, PRA started to increase (Fig. 1). The PRA continued to rise in all animals but was slightly higher in the non-lactating goats until the end of the dehydration period, when the mean values of PRA in the lactating goats exceeded those seen in the non-lactating ones. The rise in PRA was similar in the two groups of animals, if the percentage loss of plasma volume was considered, whereas the PRA rose more in relation to the loss of body wt in the non-lactating than in the lactating animals. Thus, at a loss of 23% of the initial body wt, the PRA exceeded 3 ng/ml/hr in the non-lactating goats, but was only 2.1 ng/ml/hr when the lactating goats had lost 26% of their body wt.

Rehydration

After 4 days of water deprivation the animals were allowed to drink water to satisfaction. Lactating animals immediately drank 7.2 ± 0.7 l and non-lactating ones 6.8 ± 0.1 l. In the blood samples taken within 2 min after completion of drinking, there was no change either in pOsm or pNa (Fig. 1). Plasma K concentration was 4.3 ± 0.3 mM/l in lactating goats and 4.5 ± 0.1 mM/l in non-lactating ones. The PRA remained elevated, but the pAVP concentration fell markedly (Fig. 1). The next blood samples were taken 60–80 min after drinking. The pOsm had still not decreased in non-lactating animals, but had done so in the lactating goats. Also a minor decrease in pNa was observed in both groups of animals (to 156 ± 2 and 157 ± 1 mM/l). The plasma K concentration was 4.0 ± 0.1 and 4.0 ± 0.1 mM/l respectively. At this time, the PRA reached the highest values noted during the experiment (Fig. 1). The pAVP concentration had fallen considerably in the non-lactating goats, but remained above 20 pg/ml in the lactating animals. In the final blood samples which were taken 4–4.5 hr after drinking, pOsm and pNa had fallen almost to pre-dehydration values in the non-lactating goats, but were still considerably elevated in the lactating goats. The pK concentration now reached its highest level in both groups of animals or 4.5 ± 0.1 mM/l in lactating goats and 5.2 ± 0.6 mM/l in non-lactating ones. Plasma AVP concentration had returned to the basal level in the non-lactating goats, but remained elevated in the lactating ones, PRA was still high in both groups of animals (Fig. 1).

Hemorrhage

All animals were bled until forced breathing began to appear. This did not occur until more than 23% of the initial blood volume had been withdrawn from two of the non-lactating animals, and was still hardly noticeable in the third non-lactating goat at this volume loss. However, bleeding was stopped in that animal when 28% of the blood volume had been removed. No release of vasopressin was seen in one of the animals (bled 23%), a rise to 11 pg/ml occurred in one animal (bled 28%) and a rise to 35 pg/ml in the third (bled 25%) (Fig. 2). Fifteen minutes after cessation of bleeding, the pAVP was moderately increased in all three animals. It continued to increase in one of the animals 45 min after the end of bleeding, but declined thereafter in all animals. Except for an increased respiratory rate (50–100 breaths/min), the animals did not seem disturbed by bleeding. They ate or ruminated during the whole post-bleeding observation period. The measurement of plasma volume which was made 2.5–3 hr post-bleeding revealed that the animals had

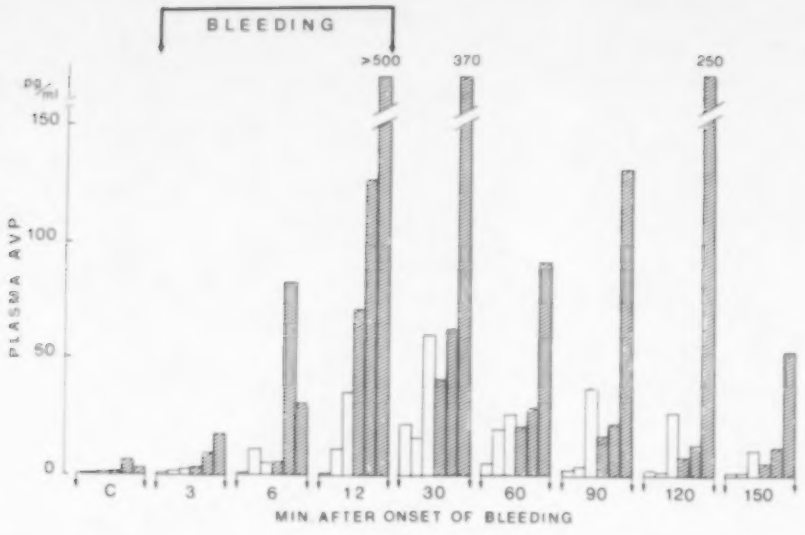

Fig. 2. Effects of bleeding on the plasma vasopressin concentration. Open bars = lactating goats. Shaded bars = non-lactating goats. Each goat is represented in the same position relative to the other animals at all times. C = pre-bleeding values (mean of three samples from each goat).

not compensated for their volume loss at this time (Table 2). The animals had had no access to water during the day. Yet none of the animals drank when they were offered water at the end of the experiment, although the plasma osmolality had risen by 13 ± 1 mOsm/kg during the day.

Hemorrhage in lactating goats caused a more serious reaction, although these animals also were relatively tolerant to bleeding. In the three lactating goats, the bleeding was stopped after 21, 22 and 27% of blood withdrawal because of respiration rates above 80/min. In the animal bled of 27% of its initial blood volume, pAVP had increased to above 500 pg/ml. At this time, the pAVP conc. in the other two animals was 60 and 125 pg/ml respectively (Fig. 2). In one of the animals the hematocrit fell to 13% and the second plasma volume measurement was therefore not performed. Instead, the blood was returned, after which she drank 3.8 l of water (her plasma osmolality had increased by 18 mOsm/kg during the day). Plasma volume had not recompensated after 2.5–3 hr in the two other lactating goats (Table 2). Although the animals had had no access to water since the previous afternoon, one of them did not drink at all (increase in plasma osmolality during the day: 7 mOsm/kg), whereas the third lactating goat drank 1.2 l (increase in plasma osmolality: 6 mOsm/kg). The animals delivered the normal amount of milk after these experiments.

DISCUSSION

In comparison to previous studies on Bedouin goats (see Introduction) the initial plasma volumes were comparatively low in both lactating and non-lactating goats used in this study. However, when these goats were deprived of water for 4 days, the lactating goats maintained their plasma volume/body wt relationship, in contrast to the non-lactating goats. The increase of pOsm, pNa and pAVP were similar in all animals. Therefore, it appears that lactating black Bedouin goats maintain their plasma volume by withdrawal of isotonic fluid from other body fluid compartments.

PRA or plasma renin concentration increase in response to dehydration in man (Maebashi and Yoshinagn, 1967), rats (Gross *et al.*, 1965; Rosenthal *et al.*, 1969), sheep (Blair-West *et al.*, 1972) and goats (Olsson *et al.*, 1978; Olsson *et al.*, 1982). In this study, the PRA did not increase in the lactating animals until 13% of the plasma volume had been lost. At this stage,

Table 2. The blood loss and plasma and blood volumes before and 2–2.5 hr post-bleeding are presented. Goats Nos. I, II, III were non-lactating, goats IV, V, VI were lactating

Goat	Body wt	Plasma volume (ml)		Blood volume (ml)		Amount bled		
		Before	After	Before	After	(ml)	(% of blood volume)	(% of body wt)
I	24.1	1550	1558	2067	1846	480	23	20
II	20.5	1069	910	1444	1124	400	28	20
III	27.5	1512	1253	1900	1474	480	25	18
IV	27.0	1861	1681	2386	2040	510	21	19
V	27.0	1875	—	2315	—	500	22	19
VI	19.5	1022	856	1470	1092	400	27	21

the non-lactating goats had lost 23% of their plasma volume which may explain why the PRA was higher in these animals (Table 1 and Fig. 1). The PRA of the lactating animals was slightly lower in all blood samples taken during dehydration, until the last blood sample taken before the animals got water. In goats, the normal increase in blood flow to the udder at the onset of lactation is achieved mainly by local vasodilation (Linzell, 1974). A delayed and reduced increase in PRA probably prevents a rapid rise of the potent vasoconstrictor angiotensin II, which would cause vasoconstriction in the udder. A reduction in milk production could thus be prevented. In support of this theory is the fact that the milk yield was almost unaffected during the first 48 hr in this as well as in previous studies (Maltz and Shkolnik, 1980) and during this time there was only a minor rise in PRA in the lactating animals.

In man, a close correlation between pAVP and pOsm has been demonstrated (Robertson *et al.*, 1976), and a similar correlation has been shown to exist also between pNa and pAVP in goats (Olsson *et al.*, 1978). In our experiments, the water loss was accompanied by marked increases in pOsm and in pNa concentration by the first evening in all animals, but thereafter, these variables increased only slowly. In contrast, the pAVP concentration did not start to increase until the second evening (Fig. 1), and marked increases in pAVP were not seen until during the third and fourth day of dehydration.

AVP at high levels is a potent vasoconstrictor agent particularly of the vessels to the gastrointestinal tract (Saamali, 1978). It may be suggested that the delayed, modest increase of the pAVP, especially in the lactating Bedouin goats, prevents a reduction of the blood supply to the gastrointestinal tract, enabling the animals to continue to eat and digest food. It is likely, that this is even more true in other Bedouin goats, since they normally keep a higher plasma volume than the goats used in this study. Furthermore, AVP in high doses (Konar and Thomas, 1970) but not in low doses (Peaker and Linzell, 1973) has been shown to reduce milk production. Thus the delayed increase of PRA and of AVP during water deprivation could be the reason why Bedouin goats can continue to produce almost normal amounts of milk despite periods of water shortage.

Following drinking that ended dehydration, all goats regained their initial body wt during one continuous drinking session, which is in agreement with previous dehydration experiments (Choshniak and Shkolnik, 1977b; Maltz and Shkolnik, 1980). During the first minutes following drinking, pOsm and pNa remained elevated, whereas pAVP concentration droped markedly, especially in the non-lactating goats (Fig. 1). Similar effects have been reported in dogs (Thrasher *et al.*, 1981), which made these authors conclude that oropharyngeal factors account for the rapid inhibition of the AVP release. In support of this conclusion, is the findings by Vincent *et al.* (1972) that the neurosecretory cells of the supraoptic nucleas are inhibited when monkeys consume water. In the non-lactating Bedouin goats, pAVP had returned to basal levels 4–4.5 hr post-drinking. However, pOsm was still elevated at this stage, which supports the observation that plasma volume is not yet back to normal (Choshniak and Shkolnik, 1977b). Also in lactating goats the pAVP concentration dropped immediately after drinking, but the values were around 20 pg/ml 90 min post-drinking, and 4–4.5 hr post-drinking they were still as high as around 12 pg/ml. The pAVP values corresponded to the slow decrease of the pOsm in the lactating goats. The delayed return to basal levels of plasma volume, pOsm and pAVP concentration upon rehydration is by all probability due to the modest absorption of water from the rumen to the plasma (Choshniak and Shkolnik, 1977b).

In agreement with previous findings in sheep (Blair-West *et al.*, 1972; Blair-West et al., 1979) and goats (Olsson *et al.*, 1978), PRA did not fall, but increased after rehydration. In the sheep experiments, it was suggested that the rise in renin concentration after rehydration was due primarily to a reduced pK. However, in this study the pK increased after rehydration. Urine flow is very low after rehydration in the Bedouin goats (Choshniak and Shkolnik, unpublished observations) and it has been suggested that an altered sodium transport at the macula densa may be the stimulus for renin release. Recent experiments in the lactating Swedish goat showed that after 48 hr dehydration the sight of the water bucket caused an immediate, pronounced increase in arterial blood pressure and heart rate, which persisted during the drinking session. The blood pressure remained elevated for about 1 hr post-drinking (Olsson K., unpublished observations). One of the stimuli for renin release (Ganong, 1981) is sympathetic stimulation, and it is possible that the rise in PRA immediately after drinking may have been due to such a stimulation. The combination of an increased sympathetic tone and the persistently reduced plasma volume may explain the slow return of PRA to basal values after rehydration.

Changes in plasma volume have been shown to shift the osmotic threshold for AVP release (Robertson and Athar, 1976). Yet the plasma pAVP concentrations seen during dehydration involving a plasma volume loss of 32–34% with accompanying hyperosmolality are in the range of, or considerably lower, than those obtained after a blood loss of 21–28% (Figs. 1 and 2). These results therefore strengthen the hypothesis made by Arnauld *et al.* (1977) and Larsson *et al.* (1978) that it is not hypovolemia *per se*, which causes the huge pAVP release during hemorrhage but rather the fall in arterial blood pressure. Although, the arterial blood pressure was not measured in this study, forced breathing was seen in all animals at the cessation of hemorrhage, indicating that a fall in arterial blood pressure indeed had occurred. The forced breathing is also compatible with the idea that hypoxia may contribute to the large AVP release which is seen during hypotensive hemorrhage (Forsling and Ullman, 1976). An alternate explanation for the difference between the magnitude of AVP release after hemorrhage and after dehydration may be the existence of receptors sensitive to rate of volume change. Thus, during the rapid blood withdrawal, the loss of plasma volume could not be replaced immediately by fluid from other body compartments. Then, lactating goats responded with a rapid and pronounced increase of pAVP, whereas non-lactating goats, which apparently were better prepared to withstand a dimini-

shed plasma volume, only got a comparatively small increase of pAVP.

In all animals plasma osmolality rose considerably during the day they were bled. Yet only two of the lactating goats drank at the end of the experimental day. The reason why the other four animals did not drink may be: (1) the blood which was returned to the animals had been taken from them when the plasma osmolality still was low; (2) by the time the lost blood was returned the goats had reabsorbed some of the lost volume from other body fluid compartments (Table 2), causing plasma expansion (Larsson *et al.*, 1978) after that the blood was retransfused.

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ACTIVE PYRIMIDINE ABSORPTION BY CHICKEN COLON

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Abstract—1. Pyrimidine absorption by chicken large intestine was investigated employing the everted sac and flux chamber techniques. ^{3}H-labelled uracil was used as substrate.

2. The small intestine and the colon, unlike the caecum, transported uracil from the mucosal to the serosal surface against a concentration gradient in the everted sac experiments.

3. Furthermore, there was a net transport of uracil from the mucosal to the serosal side of the colon and jejunum in the flux chamber experiments.

4. Uracil transport by the everted colon sacs against a concentration gradient was inhibited when the purine hypoxanthine was present in the incubation medium.

5. Uracil transport by the everted colon sacs was also inhibited under anaerobic conditions and when 2,4-dinitrophenol was present in the incubation medium. Replacing the Na^+ ions of the incubation medium by Li^+ ions also caused an inhibition of uracil transport.

6. It is concluded from these results that uracil (and probably other pyrimidines) are absorbed from the chicken colon by a Na^+ ion-dependent active transport process having also an affinity for purines.

INTRODUCTION

It has been demonstrated with the everted gut sac technique that pyrimidines are absorbed from the small intestine of rats (Schanker and Tocco, 1960, 1962), hamsters (Schanker and Tocco, 1962), frogs (Czáky, 1961; Schanker and Tocco, 1962), and sheep (Scharrer and Amann, 1979, 1980) by an active transport process. Furthermore, uptake of the pyrimidine base uracil across the brush border into jejunal epithelium apparently occurs by a saturable Na^+ ion-dependent transport mechanism exhibiting a relatively high substrate affinity and a low transport capacity (Scharrer and Amann, 1980).

Because it is unknown whether the large intestine is able to absorb pyrimidines, we have studied transport of the pyrimidine uracil across chicken colon and caecum with the everted sac technique (Wilson and Wiseman, 1954) and also with a flux chamber. For comparison, uracil transport across the small intestine was also studied.

MATERIALS AND METHODS

Both male and female chickens (age: 3–6 weeks), fed *ad libitum* a commercially available diet (GFK, Treff, Bayerische Kraftfutter Ges.mbH), were used for the everted gut sac experiments. For the flux chamber experiments older animals were used (age: 8–10 weeks). The animals were decapitated and then the abdomens were opened in order to remove the jejunum, the colon and the caeca. The pieces of intestine were transferred into cold oxygenated (O_2/CO_2 = 95/5) Krebs Henseleit bicarbonate buffer. In the case of the everted sac experiments (Wilson and Wiseman, 1954) the everted intestinal sacs were filled with incubation medium (=serosal fluid) and transferred to a 50 ml Erlenmeyer flask containing 10 ml incubation medium (=mucosal fluid). The gaseous phase in the Erlenmeyer flask was an O_2/CO_2 mixture (95/5). The incubation vessels were shaken (120 oscillations/min) in an incubator (38.5°C) during the experiment. After an experimental period of 45 min the everted gut sacs were removed from the incubation vessel and the substrate concentration of the mucosal and serosal fluid was determined. As substrate, ^{3}H-labelled uracil (Amersham Buchler GmbH, Brunschweig) was used. At the beginning of the experiment the substrate concentration was the same in the mucosal and the serosal fluid. The substrate concentration of the mucosal and the serosal fluid at the end of the experiment was determined from the ^{3}H-activity. In preliminary experiments we demonstrated using high performance liquid chromatography (Tiemeyer *et al.*, 1981, 1982) that the ^{3}H-activity of the incubation medium bound to uracil at the beginning of the experiments was still associated with uracil at the end of the experiments. Therefore uracil does not appear to be metabolized to any considerable extent while being transported through the intestinal wall.

As a parameter for active transepithelial uracil transport, the concentration gradient between the serosal and mucosal fluid of the everted sacs was used.

In a second series of experiments we measured the unidirectional fluxes of ^{3}H-labelled uracil across the intestinal wall, employing the experimental set-up shown in Fig. 1. In this case pieces of intestine were mounted on 1 ml plastic syringe bodies in such a way that the intestine was functioning as a barrier between two fluid compartments (A and B). The incubation medium (Krebs-Henseleit bicarbonate buffer) in compartment A (7 ml) contained ^{3}H-labelled uracil while the incubation medium in compartment B (0.5 ml) contained unlabelled uracil at the same concentration as the incubation medium in compartment A. From the appearance of ^{3}H-activity in compartment B the unidirectional fluxes across the intestinal wall could be determined. Pieces of intestine were mounted on the syringe body so that alternatively either the mucosal (m) or serosal (s) surface faced compartment B. Thus, unidirectional fluxes of ^{3}H-labelled uracil across the intestinal wall could be estimated in both directions (m→s and s→m). From the oppositely directed fluxes the net fluxes were calculated. The results are presented as means ± SEM.

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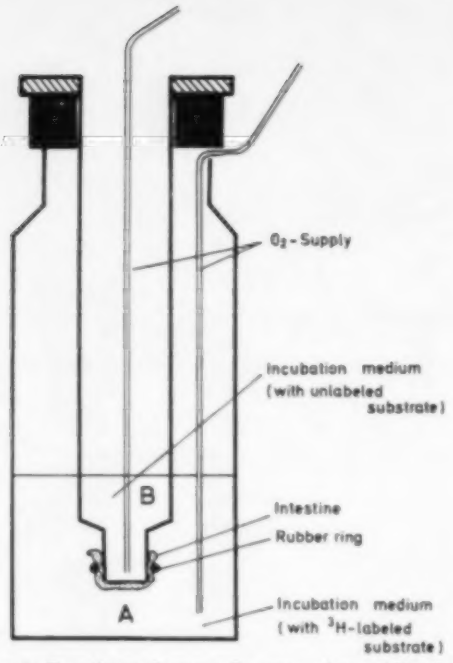

Fig. 1. Experimental set-up for measuring unidirectional fluxes of uracil across the intestine.

RESULTS

In Fig. 2 the uracil concentrations of the mucosal and serosal fluid of everted sacs of distal jejunum and colon are presented. The initial uracil concentration at both sides of the intestine was 50 μmol·l^{-1} in these experiments. During incubation there was a significant ($P < 0.001$) increase in the substrate concentration of the serosal fluid in both the jejunal and colonic sacs, while the substrate concentration in the mucosal fluid decreased only slightly because of the greater volume of the mucosal fluid. Thus, there was a clear-cut transport of uracil against a concentration gradient in both intestinal segments. Serosal transfer* related to 100 mg intestinal dry weight was 43 ± 9 nmol ($= \bar{x} \pm$ SEM) in the colon and 91 ± 12 nmol in the distal jejunum. Transport of uracil was therefore significantly ($P < 0.01$) greater in the jejunal sacs than in the colonic sacs.

*Amount of uracil transported from the mucosal to the serosal fluid.

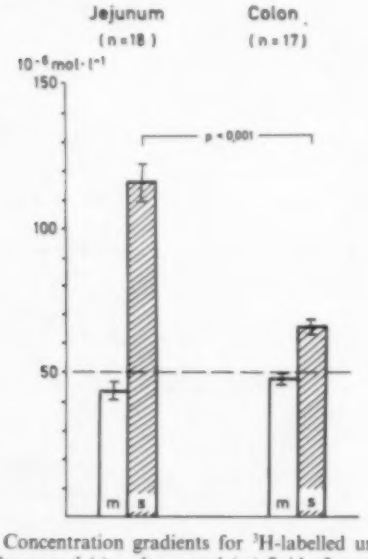

Fig. 2. Concentration gradients for ^{3}H-labelled uracil between the serosal (s) and mucosal (m) fluid of everted sacs of jejunum and colon. Initial uracil concentration: 50 μmol·l^{-1}. n = Number of animals.

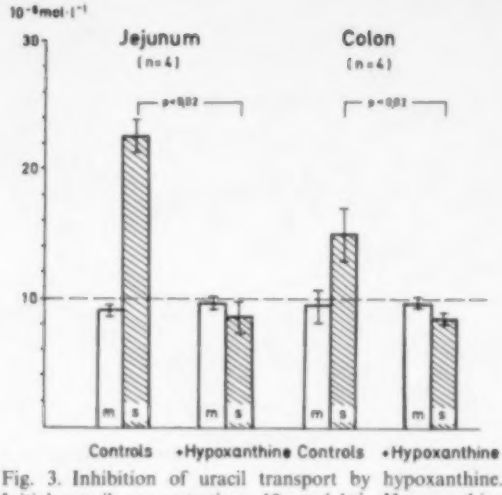

Fig. 3. Inhibition of uracil transport by hypoxanthine. Initial uracil concentration: 10 μmol·l^{-1}. Hypoxanthine concentration: 1 mmol·l^{-1}. See also legend to Fig. 2.

Figure 3 shows that in both the jejunum and colon active uracil transport was completely inhibited in the presence of the purine hypoxanthine (1 mmol·l^{-1}) in the incubation medium. The initial uracil concentrations in the mucosal and serosal fluids were 10 μmol·l^{-1} in these experiments. This result indicates that pyrimidines and purines might share a common transport system in the jejunum and colon.

We also have compared in a further series of experiments uracil transport by everted sacs of colon and caecum. The results are presented in Fig. 4. Interestingly, unlike the colonic sacs, the caecal sacs did not transport uracil against a concentration gradient. In this series of experiments we also wanted to measure the total amount of uracil taken up by the

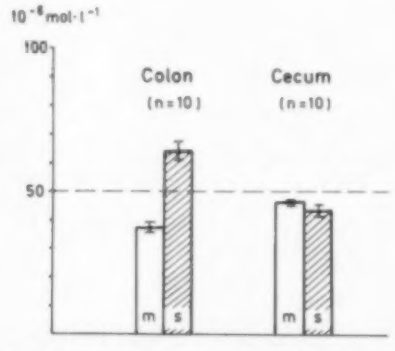

Fig. 4. Uracil transport by everted sacs of the colon and the caecum. Initial uracil concentration: 50 μmol·l^{-1}. See also legend to Fig. 2.

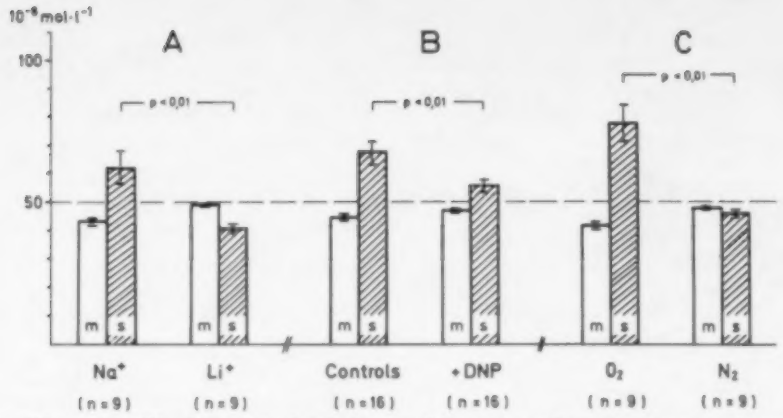

Fig. 5. Effects of modifications of the incubation conditions on uracil transport by everted sacs of the colon. A: Effect of replacing the Na^+ of the incubation medium by Li^+ on uracil transport. B: Effect of addition of 2,4-dinitrophenol (0.1 mmol·l^{-1}) on uracil transport. C: Effect of replacing the O_2 of the gaseous phase by N_2 on uracil transport. In this experiment Krebs-Henseleit phosphate buffer instead of Krebs-Henseleit bicarbonate buffer was used as incubation medium. See also legend to Fig. 2.

everted sacs from the mucosal solution (=mucosal uptake). We used larger everted sacs (volume of the serosal fluid: 1 ml) for these experiments than for the other experiments (volume of the serosal fluid: 0.4–0.5 ml) in order to get a greater decrease in the uracil concentration of the mucosal fluid during the incubation period. Moreover, the incubation time was prolonged from 45 to 60 min. The values obtained for mucosal uracil uptake by the colon and caecum sacs related to 100 mg intestinal dry weight were 102 ± 15 nmol and 47 ± 6 nmol (difference significant, $P < 0.002$). Thus, although uptake was much lower in the caecum, uracil was also taken up by the mucosa of the caecum. In spite of this finding there was no transport of uracil against a concentration gradient into the serosal fluid. Mucosal uptake of uracil by the caecum therefore probably occurred mainly by diffusion.

Figure 5 shows that active uracil transport by everted colon was inhibited under anaerobic conditions, and when 2,4-dinitrophenol, an inhibitor of oxidative phosphorylation, was present in the incubation medium. This was also the case when the Na^+ ions of the incubation medium were replaced by Li^+ ions (Fig. 5). Thus, active uracil transport by colon epithelium appears to depend on the integrity of the aerobic metabolism and the Na^+-ion-gradient across the cell membrane of the epithelial cells.

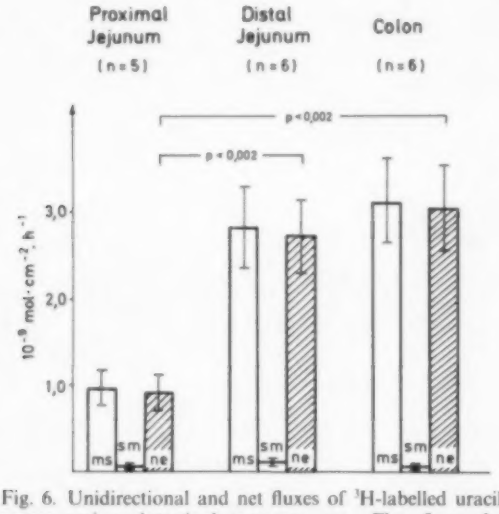

Fig. 6. Unidirectional and net fluxes of ^{3}H-labelled uracil across various intestinal segments. ms = Flux from the mucosal to the serosal side. sm = Flux from the serosal to the mucosal side. ne = Net flux.

In Fig. 6 the unidirectional fluxes of uracil across different intestinal segments are shown. The oppositely directed fluxes were determined under steady state conditions using the preparation shown in Fig. 1. Steady state values were reached after about 40 min. Therefore the 40–100 min period was used for measuring unidirectional fluxes from the mucosal to the serosal (ms) and from the serosal to the mucosal (sm) side of the intestine. It is evident from Fig. 6 that the ms fluxes were very much greater than the sm fluxes. Thus, there was a clear-cut net flux from the mucosal to the serosal side of the intestine. Flux measurements were made in the proximal and distal jejunum and in the colon. Ms-fluxes and net fluxes were significantly smaller in the proximal jejunum than in the distal jejunum and colon. Between the distal jujunum and the colon there were no differences. The latter finding is not in accordance with the results obtained with the everted sac technique (cf. Figs 2 and 6), because in everted jejunal sacs higher uracil concentration gradients between the serosal and mucosal fluid, and greater values for serosal uracil transfer, were obtained than in the colonic sacs (Fig. 2). This discrepancy might be due to the heterogeneity of the animals. The animal used for the flux experiments were considerably older (age: 8–10 weeks) than the animals used for the everted sac experiments (age: 3–6 weeks).

DISCUSSION

The results presented show that the chicken colon epithelium has the property to transport pyrimidines by a mechanism depending on the integrity of the oxidative metabolism and the presence of Na^+ ions

from the lumen to the blood side against a concentration gradient. We used the pyrimidine uracil as model substrate, because it is well known from comparable studies with the small intestine that uracil shares the same transport system with other pyrimidines, e.g. thymine and cytosine (Schanker and Tocco, 1960, 1962; Scharrer and Amann, 1980). The results obtained with everted chicken jejunum are similar to findings concerning pyrimidine absorption from the small intestine in other species (Schanker and Tocco, 1960, 1962; Scharrer and Amann, 1979). It is also interesting that the purine base hypoxanthine acted as a strong inhibitor of uracil transport both in the colon and in the jejunum of the chicken. Similar obsevations have also been made in the small intestine of other species (Schanker and Tocco, 1960; Schanker *et al.*, 1963; Scharrer and Amann, 1980). In the small intestine pyrimidines also inhibit the transport of hypoxanthine (Scharrer *et al.*, 1981). Thus, both in the small intestine and in the colon, pyrimidines and purines appear to compete for a common transport site. Interestingly, in our experiments with chicken large intestine only the colon but not the caeca showed the capability of active Na^+-ion-dependent transepithelial uracil transport. The presence of an active pyrimidine transport system in the chicken colon might have something to do with the excretion of small amounts of uracil in the urine, because in birds part of the urine flowing from the ureters into the coprodeum is transported by antiperistalsis to the colon and caeca (Akester *et al.*, 1967; Björnhag, 1981). We analysed the urine obtained from cathers in the ureter for uracil in two chickens using high performance liquid chromatography (Tiemeyer *et al.*, 1981, 1982) and found 0.31 and 0.42 $mmol \cdot l^{-1}$ uracil in the urine. Some of the uracil in the urine might therefore be actively reabsorbed by the colon epithelium. Because there are some indications that in birds the microorganisms in the caeca rapidly decompose N-containing organic compounds like uric acid and maybe also pyrimidines to NH_3 and CO_2 (Mortensen and Tindall, 1981), the absence of a mechanism for active transepithelial pyrimidine transport from caecal epithelium might be due to the lack of the respective transport substrates.

Of course, similar to the pyrimidine transport system in the small intestine, the pyrimidine transport system of the colon could also be important for the absorption of pyrimidines resulting from the digestion of dietary and endogenous nucleic acids.

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ABSORPTION AND METABOLISM OF BUTYRIC ACID IN RABBIT HIND GUT

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(*Received* 28 *March* 1983)

Abstract—1. Butyrate absorption in the large intestine of the rabbit was evaluated by the variation of concentrations in the bowel, the arterio-venous plasma and the intestinal loops. The metabolic transformations were studied with (3-4 C^{14}) butyrate.

2. The caeco-colonic epithelium oxidized negligible quantities of butyrate to ketone bodies and other metabolic pathways were found. These pathways were of different intensity according to the region of the gut and both phases of the excretory cycle.

3. A portion, which may be large, was metabolized in the caeco-colonic wall and in the liver where radioactivity was found in free amino acids, carboxylic acids and sugars. The oxidation to CO_2 in TCA cycle yields energy for metabolic activities.

4. This study of metabolism takes account of the endoflora participation.

INTRODUCTION

In an earlier work on the production and absorption of the fatty acids produced by the caeco-colonic microflora in rabbit (Vernay and Raynaud, 1975) we found, as did Hoover and Heitmann (1972), that the formation of butyrate and its role as a source of energy are different from what has been described in ruminants. High proportions of butyrate in the venous blood have been noted by other authors and were usually interpreted as the result of diffusion through the caeco-colonic wall together with the production of small quantities of acetoacetate and β-hydroxybutyrate (Cools and Jeuniaux, 1961; Henning and Hird, 1972bc; Beauville *et al.*, 1974; McMillan *et al.*, 1975; Bonnafous and Raynaud, 1978a; Woodnutt and Parker, 1981).

For more precision, we thought it necessary to experiment on the whole rabbit hind gut, studying the change in concentrations during transit, the concentrations in the arterio-venous blood and the metabolic transformations in the caeco-colonic wall and liver. The nutritive value of the butyric acid molecule is estimated from a study of metabolites that may occur in the intestinal wall and liver of the animal.

MATERIAL AND METHODS

Animals and maintenance

Male rabbits, *Oryctolagus cuniculus*, with an average weight of 2.5 kg have been used. The animals were provided *ad libitum* with oats, lucerne and water until the experiment day. The lots of rabbits were selected on the basis of their excretory cycle. When the pellets formed in the colon were hard, we used the term "day phase" and when they were soft "night phase".

Surgical procedure

The animals ($n = 20$) were anaesthetized with pentobarbital sodium (20 mg/kg in 2 ml saline NaCl 9‰). Samples of blood and of caeco-colonic content were taken at the zones specified by Bonnafous and Raynaud (1978a): one sample in the caecum, three in the proximal colon and two in the distal colon. The blood was collected in a heparinized beaker, deproteinized by perchloric acid and frozen. The gut content was homogenized, weighed and divided into two fractions: the first was frozen for later analysis, the second was incubated (38°C) for 2 hr under nitrogen. At the end of experiment this material was frozen. From other anaesthetized rabbits ($n = 36$) loops were made on the caecum (median zone), on the proximal colon (zone 3) and on the distal colon (zone 4). Each animal so prepared was put into a thermoregulation chamber. The test solution (Leng and Hörnicke, 1975) was heated to 38°C and 5 ml was introduced into the caecal and colonic loops and maintained during 20 min. The loops were previously cleaned with an isotonic NaCl solution. A non-absorbable marker substance polyethylene glycol, 2 g/l, was added to the test solution. For the metabolism studies ($n = 8$), 30 μCi of (3.4 C^{14}) butyrate was added to the test solution. At the end of experiment, the blood (arterial and venous) was collected and the rabbit was killed. The intestinal loops were emptied and carried off, a piece of each lobe of the liver was removed. These tissues were dropped into ice.

Influence of water and sodium absorption were studied by injections of antidiuretic hormone (Postacton Ferring) via the marginal vein of the ear (2.5 UI/kg), and intramuscular injections (30 μg/kg) of aldosterone (Serva Feinbiochemica).

Development of the endoluminal flora

The walls of the three zones where the intestinal loops were usually made, were removed under aseptic conditions and washed in several baths of sterile water. When the tissue was free of all digestive material, the mucosa was gently scraped and the resulting fragments, carrying the endoflora, were put in a flask containing Schaedler sterile medium (BioMérieux). This sterile medium was used for the culture of anaerobic bacteria. The addition of butyrate (15 mEq/l) and of radioactive (3.4 C^{14}) butyrate (10 μCi per flask) was performed under rigorous antiseptic conditions. The flasks were maintained for four days at 38°C.

Analytical methods

After decongelation, the samples of blood, gut contents and test solutions were centrifuged for 2 min (Eppendorfzentrifuge 3200).

Volatile fatty acids VFA were determined by gas–liquid chromatography (Intersmat IGC 120 DSL) after preparation using the technique described by Rémésy (1973). Sodium and potassium were determined by flame photometry (Gallenkamp). PEG 4000 determination was achieved according to Hyden's procedure (1955). The ketonic compounds were dosed enzymatically as described by Williamson and Mellanby (1963). The tissues of caecum, colon and liver were blotted and weighed. The area of the intestinal loops were measured by planimetry. Then, the tissues were homogenized in ice-cold water using an Ultra-Turrax electric blender. Concerning the homogenates, an extraction at high temperature, in water under low pressure permitted the elimination of VFA and other volatile substances (volatile fraction). The extract was centrifuged (30 min at 8000 rev/min), the clear supernatant solution was separated from the insoluble fraction and after elimination of liposoluble substances by chloroformic extraction (chloroformic fraction), the aqueous fraction was obtained. The ^{14}C incorporation into insoluble, chloroformic, volatile and aqueous fractions was measured in a liquid scintillation counter (Packard 460 C). From the aqueous fraction, free amino acids, carboxylic acids and sugars were isolated on a cationic (Dowex 50 × 4) and an anionic (Dowex 1 × 4) exchange resin column. Bidimensional paper chromatography was carried out for development of amino acids (first migration in butanol–acetic acid–water 4:1:5 v/v; second migration in phenol saturated by water) and carboxylic acids (first migration in ethanol–ammonia 95:5 v/v; second migration in butanol–formic acid–water 4:1:5 v/v); and monodimensional and circulary paper chromatography (butanol–acetic acid–water 4:1:5 v/v) for sugars. The autoradiography was carried out by prolonged contact with photographic plates (Kodak). The radioactive spots were cut out, eluted and their amount of ^{14}C activity was determined by liquid scintillation.

The same treatment was carried out in the Schaedler medium. In this case, we have also isolated and characterized anaerobic bacteria.

Calculations

The formulas used in calculating net transport in the intestinal loops were:

$$\text{Net water transport}_{(ml)} = V_0 - V_0 PEG_0/PEG_t$$

$$\text{Net solute transport}_{(\mu Eq)} = C_0 V_0 - C_t V_0 PEG_0/PEG_t,$$

where V_0 is the volume of fluid in loop at start of the experiment (ml), PEG_0 and PEG_t are the PEG concentrations at the beginning and at the end of experiment, and C_0 and C_t are the solute concentrations (μEq/ml) at the start and at the end of experiment. Net transport values and ^{14}C incorporation in various metabolites were calculated in term of 1 g of wet tissue. The mean weights of caecal, proximal and distal colonic loops were respectively: 6.7 ± 1.34 g; 6.5 ± 1.24 g and 5.1 ± 1.32 g. The mean serosal area was 23.0 ± 1.36 cm^2. To facilitate comparison of the various amounts of VFA, electrolytes and metabolites irrespective of their concentration, percentage values were calculated. For each experimental series, mean values and standard error are given. The statistical significance of the data was determined by t-test or by paired t-test or by the Mann–Whitney test when appropriate. The significance levels are indicated as follows:

$$*P < 0.05,\ **P < 0.01,\ ***P < 0.001.$$

RESULTS

Volatile fatty acids in gut contents

If caecal material is taken as a reference, the results (Fig. 1) suggest that there are separate mechanisms for the absorption of water and VFA in the colon. This is particularly striking in the proximal colon (zone 2) when the water is secreted (day phase) whereas 40–45% of the VFA present in the caecum disappears. VFA concentrations in the caecum of rabbits during both phases of the excretory cycle are similar. When hard faeces are excreted (day phase) there is an abrupt reduction in the VFA concentration, in the proximal colon (zone 3), from 40 to 24 mEq/l. The corresponding variation for butyrate is from 6 to 1.5 mEq/l, the hard faeces contain considerably less butyrate than do the soft faeces. If we compare the production *in vitro* and the disappearance *in situ* of the three VFA in the digestive matter (Figs 1 and 2), we see that the butyrate has the highest coefficient in both cases, even though its concentration in the caecal contents (15 mEq/l) is three or four times less than that of acetate (54 mEq/l).

Volatile fatty acids in the intestinal loops

In the intestinal loops, the absorption of the VFA present in the test solution is always high (Table 1). Moreover, it is invariably greater in the proximal colon (zone 3) than in the caecum and distal colon. This difference is highly significant for the day time pattern. The rate of absorption of the fatty acids increases with the size of their molecule. Irrespective of

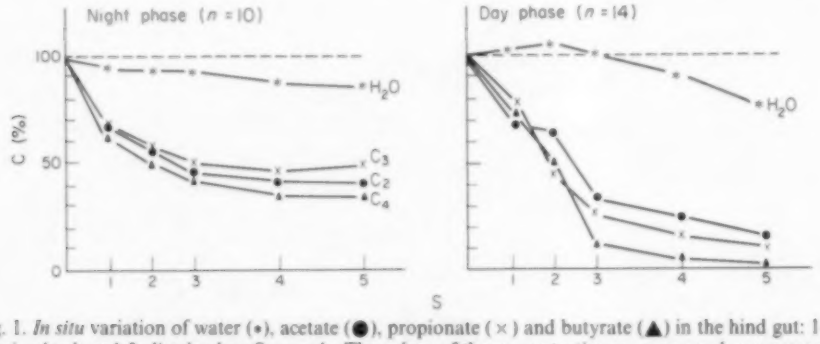

Fig. 1. *In situ* variation of water (•), acetate (●), propionate (×) and butyrate (▲) in the hind gut: 1-2-3, proximal colon; 4-5, distal colon; S, sample. The values of the concentration are expressed as a percentage of the concentration in the caecum. The standard error ranges between 0.5 and 4.5. Number of experiments (n). Unpaired t-test: comparison "night phase"/"day phase" VFA P_3C night/VFA P_3C day (significant at $P < 0.001$); VFA D_5C night/VFA D_5C day (significant at $P < 0.001$).

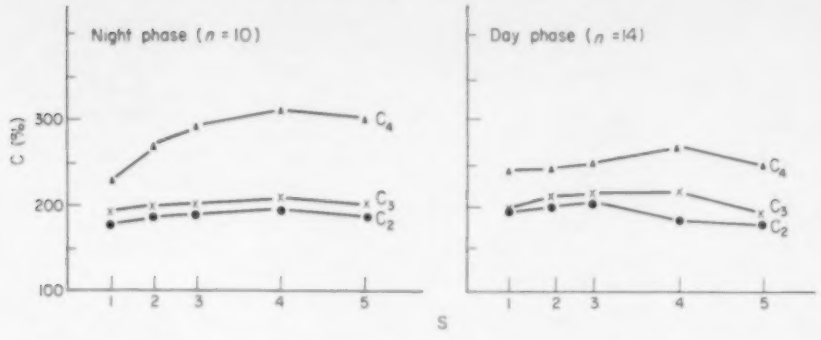

Fig. 2. *In vitro* production of acetate (●), propionate (×) and butyrate (▲) in the content of the hind gut: 1-2-3, proximal colon; 4-5, distal colon; S, sample. The values are expressed as a percentage of the initial concentration. The standard error ranges between 7 and 37. Number of experiments (*n*).

pattern and segment, 50% of butyrate inserted in the loop has disappeared after 20 min. Intravenous injection of ADH increases the water absorption, but there is no significant variation in the absorption of butyrate, sodium and potassium. In contrast, aldosterone injection provokes significant increase for all elements (Table 2).

Volatile fatty acids in the arterio-venous plasma

Measurement of the VFA in the venous plasma from the caecum and the colon reflects both phases of the excretory cycle for all the caeco-colonic segments except zone 3 (Fig. 3). For this zone, there is little butyrate released by the tissue during the "day phase". The appearance of ketonic compounds in the caeco-colonic veins is not modified by the excretory cycle. The plasma concentrations are low, the mean value of acetoacetate and β-hydroxybutyrate is respectively: 5.8 ± 0.84 and 10.6 ± 2.07 μEq/100 ml.

Butyrate metabolism

Twenty minutes after the introduction of (3-4 C^{14}) butyrate in the intestinal loops, radioactivity is studied in different tissues (caecum, colon, liver). After preparation of the fractions, the labelling balance shows that 9–16.5% of the radioactivity introduced is recovered (Fig. 4), the highest values are obtained during the "day phase". Nearly half of this recovered radioactivity is in the aqueous fraction during the "night phase" and 75% during the "day phase". These values show the importance of studying the aqueous fraction, although the variations of the labelling in the volatile, chloroformic and insoluble fractions are also of interest.

In the aqueous fraction, the labelling is distributed between free amino acids, carboxylic acids and sugars. The homogeneity of the chromatograms for each experiment must be noted (Table 3). The labelled amino acids are principally: aspartic acid, glutamic acid, arginine, histidine and lysine; but alanine, glycine and amino acids of the glutamic acid family are also found, mostly in the "day phase". In the distal colon extracts methionine and valine are detected. The labelled carboxylic acids are more numerous during the "day phase". In this case most of the ^{14}C is found in citric acid, succinic acid and lactic acid, and to a lesser extent, in glycolic acid, other TCA cycle acids and adipic acid. Radioactive sugars are found on the

Table 1. Net absorption of water and volatile fatty acids in the intestinal loops

	Night phase Intestinal loops (n = 18)			Day phase Intestinal loops (n = 18)		
	CC	P_3C	D_4C	CC	P_3C	D_4C
H_2O	16.7 ± 2.80	12.4 ± 2.14	8.3 ± 2.87	19.8 ± 2.12	22.0 ± 2.77 *	19.0 ± 3.33 *
Acetate	26.7 ± 3.12	27.3 ± 2.27	26.0 ± 2.80	29.4 ± 3.18	44.3 ± 3.68 ***	32.5 ± 2.49
Propionate	38.9 ± 3.04	41.5 ± 2.19	31.9 ± 3.28	42.8 ± 3.86	54.0 ± 2.88 **	44.3 ± 1.70 **
Butyrate	42.8 ± 3.09	45.7 ± 2.03	43.6 ± 1.46	47.0 ± 4.35	58.4 ± 3.28 **	50.2 ± 2.66 *
Total	32.7 ± 3.08	34.9 ± 1.50	33.0 ± 1.87	35.9 ± 3.18	48.3 ± 2.72 ***	39.2 ± 1.38 *

The net absorption is expressed as a percentage of the initial concentration of the test solution in the intestinal loops. CC, caecal loop; P_3C, proximal$_3$ colonic loop; D_4C, distal$_4$ colonic loop. Number of samples (n). Unpaired t-test: comparison "night phase"/"day phase".

*Significant at $P < 0.05$.

**Significant at $P < 0.01$.

***Significant at $P < 0.001$.

Table 2. Action of antidiuretic hormone (ADH) and aldosterone on the net absorption of water, electrolytes and volatile fatty acids in the proximal colonic loops

	Controls ($n = 8$)	Aldosterone ($n = 8$)	Controls ($n = 8$)	Antidiuretic hormone ($n = 8$)
H_2O	18.1 ± 3.73	31.4 ± 8.4 **	13.3 ± 5.95	47.7 ± 13.7 **
Sodium	40.4 ± 9.56	57.2 ± 7.91 *	30.2 ± 5.84	36.7 ± 7.45
Potassium	32.5 ± 3.30	55.6 ± 6.28 *	24.5 ± 7.32	35.1 ± 6.96
Total VFA	64.0 ± 2.95	78.8 ± 3.43 **	37.7 ± 3.35	51.7 ± 4.66 *
Butyrate	75.8 ± 0.16	86.9 ± 1.32 ***	66.3 ± 2.97	66.8 ± 3.61

The net absorption is expressed as a percentage of the initial concentration of the test solution in the proximal colonic loops. Number of samples (n). Paired t-test: comparison control animals/test animals.
*Significant at $P < 0.05$.
**Significant at $P < 0.01$.
***Significant at $P < 0.001$.

autoradiograms of the caeco-colonic neutral fraction: glucose, fructose, maltose and sucrose. These sugars are labelled with the same intensity. Phosphorylated monosaccharides are most abundant during the "day phase". In the autoradiograms of liver neutral fraction, only phosphorylated monosaccharides and glucose are found. The highest values, obtained during the "day phase", are due to the increased labelling of

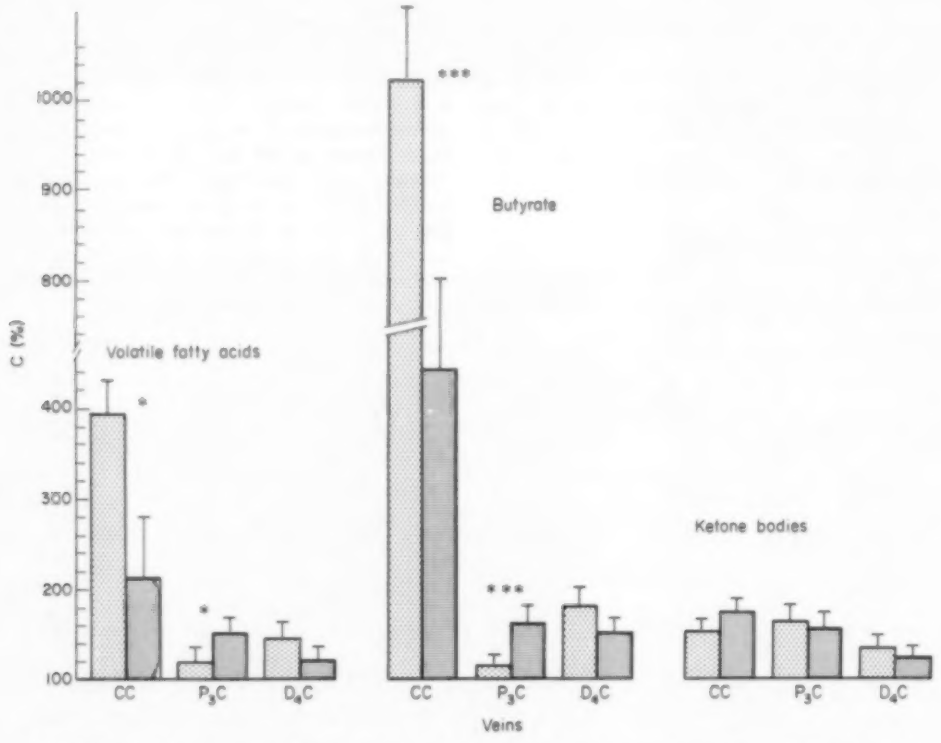

Fig. 3. Comparison of the variations of the total volatile fatty acids, butyrate and ketone bodies in the efferent plasma as a function of the excretion pattern. For each of these the left hand column represents "day phase" ($n = 6$) and the right hand column represents "night phase" ($n = 6$). The values of the concentration in the venous plasma are expressed as a percentage of the concentration in the corresponding artery. CC, caecal vein; P_3C, proximal$_3$ colonic vein; D_4C, distal$_4$ colonic vein. Number of experiments (n). Unpaired t-test: comparison "night phase"/"day phase". * Significant at $P < 0.005$. Significant at $P < 0.001$.

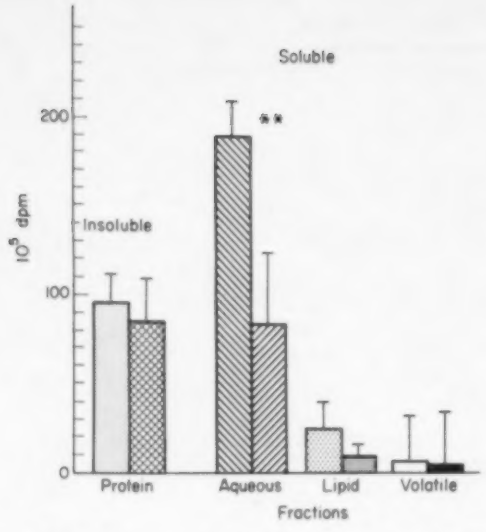

Fig. 4. Total incorporation of ^{14}C in the different biochemical fractions as a function of the excretion pattern. For each fraction left hand column represents "day phase" ($n = 16$) and right hand column "night phase" ($n = 16$). The values of ^{14}C incorporation are expressed in 10^5 dpm for the totality of tissues studied: liver (110 g), caecum (36 g) and colon (50 g). Number of experiments (n). Insoluble: protein. Soluble: aqueous fraction; chloroformic fraction; volatile fraction. Mann–Whitney test: comparison "night phase"/"day phase". **Significant at $P < 0.01$.

amino acids and carboxylic acids which are 12 times more labelled for the caecum, 15 for the proximal colon and 18 for the distal colon. In hepatic tissue during the "day phase" there is an increase of amino acids and sugars (Fig. 5).

In vitro *study of the endoluminal flora*

The experimental procedure, whereby the caeco-colonic endoflora was isolated, has the advantage that the microbial population within the crypts of the mucosal is relatively unaffected. We found *Bacteroïdes* and *Clostridium*, anaerobic Cocci, at a density of 10^4–10^5/ml for each group. We found no Enterobacteria or Lactobacilli. In the culture medium basic amino acids (arginine, histidine, lysine) and succinic acid accumulate, but in our experiments only adipic acid is labelled.

DISCUSSION

Allocation of the rabbits into two batches according to the nature of the pellets in the hind gut, is the only way of obtaining a consistent, statistical interpretation of the experimental results (Henning and Hird, 1972a; Bonnafous, 1973). The biochemical study reveals that the variations alternate with the excretion pattern. The physiological phenomena studied are intensified during the "day phase" (Figs 1, 3, 4 and 5). The high rate of production of butyrate (Fig. 2) is probably related to the very unusual nature of the microflora species in the rabbit, characterized by

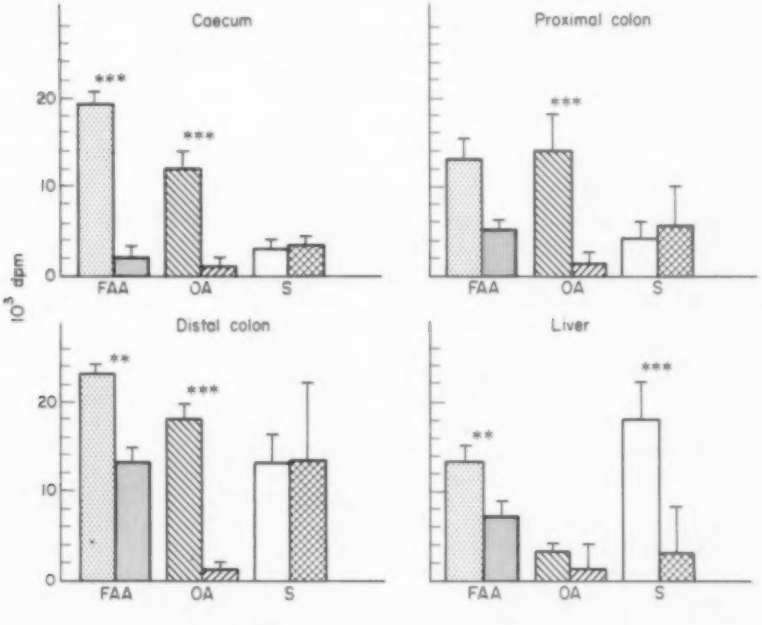

Fig. 5. Incorporation of ^{14}C in free amino acids (FAA), organic acids (OA) and sugars (S) in relation with the excretion pattern. For each of these the left hand column represents "day phase" ($n = 4$) and the right hand column represents "night phase" ($n = 4$). The ^{14}C incorporation is expressed in 10^3 dpm/g wet tissue. Number of experiments (n). Mann–Whitney test: comparison "night phase"/"day phase". **Significant at $P < 0.01$. ***Significant at $P < 0.001$.

Table 3. Incorporation of ^{14}C in metabolites of the aqueous fraction, after introduction of (3.4 C^{14}) butyrate in the intestinal loops

	Day phase ($n = 4$)				Night phase ($n = 4$)			
	CC	P_3C	D_4C	L	CC	P_3C	D_4C	L
Bu-NH_2	+	+	+	+				+
Ala	+ +	+	+	+ +				+
Asp	+	+	+ + +	+ +		+ + +	+ + +	+
Glu	+ + +	+ + + +	+	+ +		+ +	+	+ + +
Gln		+	+	+ +	+ + +			
Gly	+	+	+	+ +	+		+ +	+ +
Ser								
%	67.3 ± 6.50	96.3 ± 1.81	79.9 ± 15.61	86.5 ± 2.50	100	87.0 ± 9.69	73.5 ± 1.13	92.7 ± 9.72
Arg	+ +	+	+ +			+ +	+ +	+
His	+ +	+	+	+			+ +	
Lys	+			+ +				+
Cy-SH	+ +	+	+ +	+ +			+ +	+
Tyr							+	
Val							+	
%	32.7 + 6.50	3.7 ± 1.81	20.1 ± 15.61	13.5 ± 2.50		13.0 ± 9.69	26.5 ± 1.13	7.3 ± 9.72
Cit	+ +		+ + +	+	+		traces	+
Fum		+	+ +	+				+
Mal	+ +		+	+ +	+			+ +
OxA	+		+					
αGlu	+		+	+				
Succ	+ +	+ +	+	+ +	+ + +	traces		+ +
%	75.7 + 6.76	47.1 ± 6.71	63.5 ± 15.02	73.7 ± 1.22	81.3 ± 18.3			69.2 ± 43.50
Adip	+ +		+ +	+				
%	15.5 ± 2.40		13.9 ± 7.64	5.6 ± 7.25				
Gly	+	+	+ +	+	+			+
Lact	+	+ +	+	+	+			+
%	8.8 ± 2.17	52.9 ± 6.72	22.5 ± 7.38	20.7 ± 6.63	18.7 ± 10.02			30.8 ± 43.50
P. os.	+ + +	+ + +	+ +	+ + +		+	+	+ +
Fruct	+	+	+		+	+	+	
Glu	+	+	+	+	+	+		+ + +
Malt		+	+		+			
Sucr.		+	+					

The ^{14}C incorporation is expressed as a percentage of the total ^{14}C incorporation of free amino acids and carboxylic acid found on the autoradiograms: $0 < + < 10\%$; $10 < + + < 20\%$; $20 < + + + < 50\%$; $50 < + + + + < 100\%$. The different elements are regrouped as a function of the metabolic pathways. For the sugars, the number of autoradiograms obtained is reduced and the percentage of the mean value is not calculated. The sign + designates the presence of the sugars. Number of samples (n).

the absence of Lactobacilli and by the predominance of strictly anaerobic species, especially *Bacteroïdes* (Fonty, 1974). Among these species there may be dominant strains, such as *Butyrivibrio*, that produce butyrate (Ørskov *et al.*, 1970). Irrespective of the excretion pattern, the VFA fermentation is always important (Fig. 2), meanwhile the density of the bacteria is higher in the "night phase" content (Bonnafous and Raynaud, 1970), but the substrates utilized for the VFA fermentation are certainly different: intra or extra bacterial sugars (Bonnafous and Raynaud, 1978b).

It has been shown by Leng (1978) that the rate of absorption of the VFA in rabbit caecal loops increases with the size of the molecule and he established correlations between the movements of the electrolytes and those of the VFA. Our results (Tables 1 and 2) show that Leng's conclusion can be generalized to include the colon: butyrate has the highest coefficient of absorption (Table 1), and that of water is unrelated to that of VFA during caeco-colic transit (Fig. 1) and in the intestinal loops after intravenous injection of ADH (Table 2). Furthermore, injection of aldosterone leads to an increase in electrolyte, water and VFA absorption (Table 2). The absorption in the colon does not diminish as the distance from the caeco-colic junction increases which might be suggested by the variations in butyrate efferent plasma concentration (Fig. 3), since the rate of disappearance of the butyrate is always important when a solution of identical concentration is introduced into the caeco-colonic loops. Moreover, it is invariably greater in the proximal colon than in the caecum and the distal colon (Fig. 1, Table 1). In contrast to ruminants in which the rumen epithelium oxidizes considerable quantities of butyrate to ketonic compounds (80%) and CO_2 (Hird and Symons, 1961; Emmanuel, 1980), in the rabbit the caecal and colonic tissue converted negligible quantities of this acid to ketonic compounds (Fig. 3). There seems to be agreement among the various authors that there is little variation among the levels of ketonic compounds in the efferent venous plasma of the large intestine of the rabbit and that plasma concentration

of β-hydroxybutyrate is very low (Henning and Hird, 1972bc; Bonnafous and Raynaud, 1978a; Woodnutt and Parker, 1981). Therefore, the presence of butyrate in rabbit hind gut raised some speculation. Henning and Hird (1972b) show a close correlation between 3-hydroxy-3 methylglutaryl CoA synthase activity and ketogenic capacity along the colon, this capacity diminished as the distance from caeco-colic junction increased. Metabolic pathways that do not involve the capacity of rabbit caeco-colonic tissue to produce ketonic compounds must be found. The absorption of radioactive butyrate is accompanied by labelling of certain metabolites in the tissue extracts. Their distribution indicates that the metabolic activity is more intense during the "day phase" (Table 3), when the butyrate is vigorously absorbed (Fig. 1, Table 1). In "night phase" there is relative accumulation of some metabolites such as: citric acid, glutamic acid and succinic acid, which is explainable if we accept that the metabolic activity drops when the butyrate acid absorption falls. In view of our experimental arguments and in particular of the heavy labelling of the carboxylic acids of the TCA cycle, it seems reasonable to ascribe the role of principal user of butyrate to the enterocyte, which employs it in its oxidative metabolism. The labelling of certain indispensable amino acids and of sucrose in the caeco-colonic extracts does, however, imply the participation of enzymes of microbial origin, as we have already observed in the acetate metabolism (Marty *et al.*, 1981). In our attempts at endoflora culture, radioactivity is found only in adipic acid. In the intestinal loops metabolites are probably exchanges between the enterocyte and the microbial cells, and this cannot be reproduced in our endoflora cultures. In the terminal regions of the gut, therefore, numerous biochemical interactions between the mucosal and the microflora of the crypts seem to occur, depending on the ambient conditions. Furthermore, we have shown that a pouch prepared from the rabbit caecum can be considered to perform physiological caecal functions only when its mucosa is provided with normal caecal microflora (Leng and Marty, 1979).

From our study of metabolism, we can conclude that butyrate is actively metabolized in the caeco-colonic tissue and the extent of the phenomenon depends on the excretion pattern. When the animal produced soft pellets ("night phase"), the absorption of butyrate and metabolism were relatively modest, when the pellets were hard ("day phase"), these were both increased. The mechanisms involved appear to be linked to hormonal effects (aldosterone). In zone 3 of the proximal colon, the mechanisms described above are intensified. Although the butyrate content falls, the enrichment of the efferent blood is not significant (Fig. 3) and a study of the intermediate metabolism shows that the butyrate is totally metabolized to CO_2 in the tissue. The originality of this zone (only a few centimetres long) is well known (Bonnafous, 1973). Perhaps, the various mechanisms involved in the liberation of the "lytic factor" and in the absorption of different bacterial constituents requires considerable energy and that the butyrate molecules yield energy for metabolic activities. The occurrence of labelled adipic acid in the liver during the "day phase" indicates the complexity of the metabolic pathway that leads the radioactive carbon of the butyrate from the caeco-colonic luminal to the liver cells. This example suggests that some metabolites recovered in the hepatic extract are probably absorbed forms of the butyrate which does not exclude an hepatic metabolism based on the butyrate uptake. This metabolism is extremely efficient in the rabbit, thus permitting a practically quantitative removal of the absorbed butyrate in the plasma portal vein (118 ± 7.1 μEq/100 ml). Only traces of this acid are recovered in the arterial plasma (14 ± 3.7 μEq/100 ml).

Table 4. Mean disappearance of volatile fatty acids in the intestinal loops and their equivalent metabolizable energy

	Absorption rate ($n = 60$) (μ equivalent/g)	Equivalent energy (calories)	(%)
Acetate	31.2 ± 2.13	6.6 ± 0.45	36.1 ± 1.27
Propionate	9.2 ± 0.48	3.4 ± 0.18	18.9 ± 0.49
Butyrate	15.5 ± 0.75	8.2 ± 0.39	45.0 ± 1.05
Total	56.0 ± 2.22	18.3 ± 0.37	100

Metabolizable energy of each VFA is calculated from the caloric value of its heat combustion. Heats of combustion of acetate, propionate and butyrate are taken as 209.4, 367.2 and 524.2 cal/μEq, respectively (Hodgman *et al.*, 1958). Number of samples (n).

The quantity of VFA that disappears in the intestinal loops enable us to evaluate the energy supplied of these acids (Table 4). Metabolizable energy of each acid is calculated from the caloric value of its heat of combustion (Hodgman *et al.*, 1958). Nearly half of the energy supplied is due to butyrate, this is in good agreement with the report of Hoover and Heitmann (1972). These theoretical figures certainly give an idea of the real values since the butyrate molecule is involved in the TCA cycle and can hence reach the final stage of oxidation (CO_2).

The importance that we attribute to butyrate as a source of energy for the mucosal of the large intestine in the rabbit may also be applicable to other species. Thus Roediger (1979, 1980), working on rat and human colonocytes, established that over 80% of energy needs for the colonic mucosa in the rat are obtained from the absorbed fatty acids, *n*-butyrate. Roediger suggests also that this fatty acid is the best respiratory fuel for the human colonic mucosa, particularly the distal colon.

SUMMARY

Of the various VFA produced by the caeco-colonic bacteria of the rabbit, butyrate differs from the other volatile fatty acids by its rate of production, its absorption and its energy supplied.

This study of butyrate shows that absorption in the caeco-colonic regions is not a simple diffusion. Complex mechanisms, which vary with the zone of the intestine studied, occur in the tissue and vary with the excretion pattern. It seems difficult to separate the caeco-colonic mucosa from the endoflora, from both the physical and biochemical stand-points.

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TEMPERATURE RESPONSES OF STANDARD, AEROBIC METABOLISM BY THE CALIFORNIA LEGLESS LIZARD, *ANNIELLA PULCHRA*

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Abstract—1. Standard metabolic rates, measured for the lizard *Anniella pulchra* at 6 and 13°C, were statistically identical, showing notable thermal-independence at low temperature.

2. Metabolic rates, measured at 25 and 30°C, were significantly less than the rates predicted by standard equations.

3. Thermal-independence and reduced aerobic metabolism at temperatures above the preferred range may result in water and energy savings in the fossorial habitat.

4. Body-mass adjusted comparisons with other lizards suggest that reduced metabolism may be a correlative adaptation in small, fossorial lizards.

INTRODUCTION

The standard (aerobic) metabolism of reptiles has been studied and related to body temperatures and other parameters for many species of lizard (see review by Bennett and Dawson, 1976). Lower than average metabolic rates of xantusiid and gekkonid lizards have been reported and discussed relative to water loss reduction and energy conservation (Snyder, 1971; Putnam, 1978; Mautz, 1979a,b; Feder and Feder, 1981; Putnam and Murphy, 1982). Since *Anniella pulchra*, the California legless lizard, is similar to the xantusiids and gekkonids in its small body size and secretive, fossorial habit, it might be expected to show similar adaptations in its metabolism.

This paper resports oxygen consumption rates for the anguimorphan, California legless lizard which inhabits coastal California and northern Baja California (Burt, 1931; Miller, 1944; Stebbins, 1964).

The California legless lizard has a relatively low preferred temperature range. Temperatures reported for lizards collected in the field ranged from 7.8 to 28.3°C with a mean value of 21.0°C (Gorman, 1957; Brattstrom, 1965). These lizards selected temperatures averaging 20.0°C in a gradient in dry sand in the lab (Brattstrom, 1965), and 24.9 and 23.6°C in moist sand (Bury and Balgooyen, 1976). Temperatures above 40°C were reported lethal by Miller (1944) and 34°C is the critical thermal maximum reported by Brattstrom (1965). Miller (1944) considered that habitat temperatures control the daily activities of these lizards.

Relatively low rates of oxygen consumption have been reported for *Anniella* (Vance, 1959; Kamel and Gatten, 1983). The current study on California legless lizards was undertaken (i) to determine rates of resting oxygen consumption at lower temperatures than heretofore studied, (ii) to compare metabolic rates of lizards buried in sand vs exposed, and (iii) to look for rates of metabolism that are lower than expected for a lizard of this size, especially at relatively high temperatures.

MATERIALS AND METHODS

Seven, adult *Anniella pulchra*, mass 3.4–6.2 g, were collected in late August, 1980 from the coastal sand dunes near Sunset State Beach, Santa Cruz County, California. The lizards were found in damp, sandy soil beneath old boards or under the litter of shrubs.

Lizards were housed in plastic shoe boxes (30 × 16 × 9 cm) in sand from their collection site. Distilled water was added to each box as needed to keep one end wet and the other dry. Ambient temperatures varied from 18 to 22°C and the photoperiod was 12 hr light, 12 hr dark. Animals were offered mealworms (*Tenebrio* larvae) and flour beetle larvae (*Tribolium*) *ad lib*. Two young born in captivity to a 4.2 g female on 7 November, 1980, weighed 0.7873 and 0.7092 g on 8 November, 1980. Metabolic rates of these two lizards were also measured.

Rates of oxygen consumption were measured during the afternoons in November and December, 1980, at various ambient temperatures in a constant pressure apparatus (similar to that described by Asplund, 1970). The chambers were 250 ml Erlenmeyer flasks, covered with black paper. Ascarite (for CO_2 absorption) and Drierite (for H_2O vapour absorption) in small mesh bags were suspended above the lizards. Data were taken for intervals ranging from 10 to 30 min, and read to 0.01 ml. An empty apparatus was run concurrently, as a thermo-barometer, to allow correction for slight variations in ambient pressure and temperature. For measurements of oxygen consumption by buried lizards, dry sand was placed on the floor of every flask to a depth of 1 cm.

Lizards used in experiments were not fed for two to three days and no food could be seen in the stomach (visible through the ventral body wall). Lizards were acclimated to the test temperature for a minimum of two days and then were placed in the apparatus 20–24 hr before the measurements were taken. Except for the two juveniles, lizards were quiescent during readings. Body temperatures were not monitored, but were assumed to be approximately equal to ambient temperature under the conditions of these experiments. The five lowest readings, out of the 20–30 taken for each lizard, were averaged and taken to represent standard metabolism. Data were corrected to STPD. Differences between means were tested for statistical significance using the one-tailed Student's *t*-test. Least squares regressions were

computed according to Sokal and Rohlf (1981) using an F-test for significance of the regression coefficients.

RESULTS

Oxygen consumption rates, for the adult lizards, generally increased with increasing ambient temperature although the Q_{10}s were different for different temperature intervals (Table 1). The overall regression equation for the raw data was:

$$\log \text{ml } O_2/\text{g/hr} = -1.729 + 0.028\ T^{\circ}\text{C},$$
$$n = 41,\ r^2 = 0.83,\ P \leq 0.001.$$

The slopes of the regression lines for log/log comparisons of oxygen consumption vs body mass were not significantly different from zero at any temperature for the adult lizards.

There was no significant difference between mean rates of oxygen consumption at 6 and 13°C or at 25 and 30°C. The mean rates of oxygen consumption did differe significantly between 13–20°C, 20–25°C, and 20–30°C ($P \leq 0.001$).

Measurements taken at 20°C, after lizards had been in the chambers for 24 hr, showed no significant difference between buried and exposed animals. Both groups had mean rates of oxygen consumption (+95% confidence limits) of 0.076 ± 0.004 (for both, $n = 9$; mean body mass ± one standard deviation equaled 4.5 ± 0.08 g and 4.4 ± 0.9 g, respectively).

The two juvenile lizards, then three days old, were tested at 20°C and the values obtained were 0.174 ml O_2/g/hr (body mass = 0.75 g) and 0.161 ml O_2/g/hr (body mass = 0.61 g).

Table 1. Oxygen consumption of *Anniella pulchra* at various ambient temperatures

T_a(°C)	n	Mass	(ml O_2/g/hr) Measured	Q_{10}	Predicted*
6	5	4.9±0.7	0.028±0.011		—
				1.0†	
13	3	5.2±0.9	0.029±0.011		—
				2.6‡	
20	18	4.5±0.8	0.076±0.004		0.071±0.001
				1.7‡	
25	6	4.7±0.7	0.099±0.013		0.131±0.004
				1.4†	
30	9	4.9±0.8	0.116±0.017		0.184±0.004

*Predicted values were calculated for each animal at 20 and 30°C from the equations of Bennett and Dawson (1976) and at 25°C from Mautz (1979a,b).
†No significant difference between values.
‡Significant difference between values ($P \leq 0.001$).
n = sample size, Mass = mean body mass in g±SD. O_2 consumption values are means ± 95% confidence interval.

DISCUSSION

The metabolic rates measured for the two new-born California legless lizards were much higher than those predicted by the equation of Bennett and Dawson (1976). Those values would be 0.102 ml O_2/g/hr and 0.106 ml O_2/hr. Bennett and Dawson (1976) have pointed out that juvenile lizards tend to remain active in metabolism chambers, as was the case in these experiments. This was the most likely cause for the elevated metabolism. The remainder of the discussion is based on data for adult lizards.

At 20°C the California legless lizard had an oxygen consumption rate which was typical for a lizard of its size. The Q_{10} for oxygen consumption between ambient temperatures of 13 and 20°C was 2.6, a value which is typical for other lizards. However, from 13 down to 6°C there was essentially no reduction in the metabolic rate ($Q_{10} = 1.04$). It is characteristic for cold-adapted lizards to show less decline in metabolic rate at low temperatures than their more heat-tolerant relatives (Dawson, 1967; Bennett and Dawson, 1976; Pough, 1976). For the California legless lizard, temperature-independent metabolism at the lower temperatures could be ecologically significant. These lizards have been found in the field, active, with body temperatures as low as 7.8°C (Gorman, 1957). A capacity for maintaining metabolism at low temperatures could benefit the lizard in foraging during the cool, wet, winter months of coastal California.

Relative independence from temperature was also apparent for oxygen consumption rates at the higher temperatures ($Q_{10} = 1.5$, 20–30°C). At 25 and 30°C, the oxygen consumption rates were significantly lower than the values predicted from the equations of Bennett and Dawson (1976) and Mautz (1979b) ($P \leq 0.001$). Templeton (1970) discussed a similar phenomenon for the collared lizard (*Crotaphytus collaris*) and the desert iguana (*Dipsosaurus dorsalis*). Grimmond and Evetts (1981) showed similar patterns of reduced Q_{10} for a skink (*Leiolopisma nigriplantare*) and a gecko (*Hoplodactylus maculatus*). The pattern of reduced oxygen consumption and lowered Q_{10} at temperatures above the preferred range is similar to that shown for the golden sand skink (*Acontias meleagris*) a legless, fossorial scincid from South Africa with a similar habitat and a similar and relatively wide preferred temperature range to the California legless lizard (Withers, 1981). The pattern of relative temperature-independence may be seen at both low and high temperatures in data for *Typhlosaurus lineatus* (Kamel and Gatten, unpublished data). This lizard is also a legless, fossorial sand-swimmer with an apparent temperature preferendum of approximately 28°C.

Since oxygen consumption rate varies with temperature and body mass, comparison of the patterns of oxygen consumption vs temperature for several species of lizard are made clearer by plotting data as log oxygen consumption in mg $O_2/g^{0.82}$/hr vs temperature (Fig. 1). This eliminates the differences in metabolic rate due to body size (Bennett and Dawson, 1976). The predicted line is probably biased upwards relative to any ideal average for all lizards in that it was calculated for a group of species which were predominately larger than 10 g body mass (10 of the 27 species used were over 100 g average body mass); and which were predominately active, diurnal lizards (17 species as opposed to eight diurnal and secretive species and one nocturnal species). The lines for the collared lizard, the anole (*Anolis carolinensis*) and the desert iguana, all heliothermic baskers with narrow thermal preferenda, are close to the predicted line. Also the line for the alligator lizard (*Gerrhonotus multicarinatus*) a taxonomically-related lizard with a broad

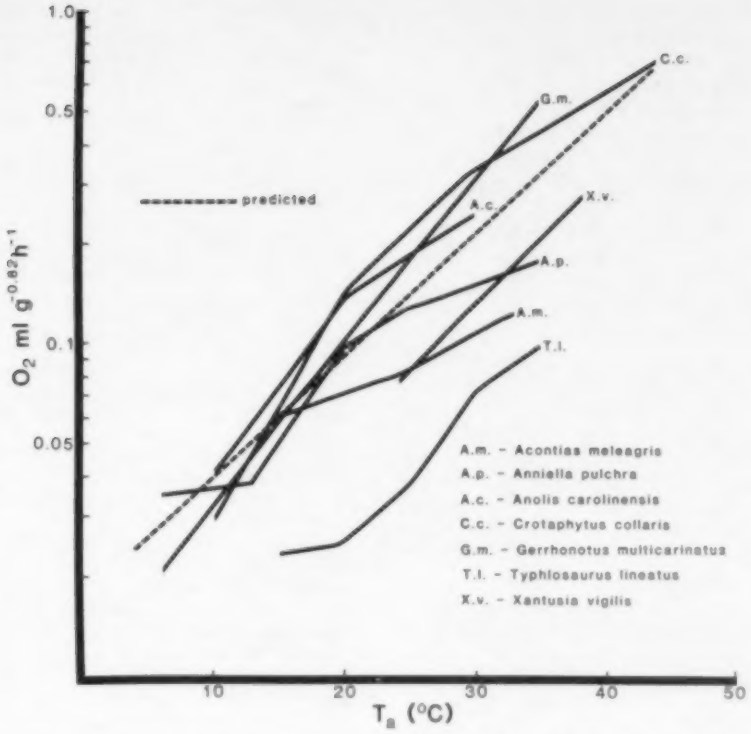

Fig. 1. Semi-log plot of oxygen consumption rates, computed relative to body mass to the 0.82 power, vs ambient temperature (T_a). Data for *Anolis* (Maher and Levedahl, 1959), *Anniella* (Vance, 1959, 35°C; present study, 6–30°C), *Crotaphytus* (Dawson and Templeton, 1963), *Gerrhonotus* (Dawson and Templeton, 1966), *Typhlosaurus* (Kamel and Gatten, unpublished data), and *Xantusia* (Snyder, 1971). Predicted line is from equations of Bennett and Dawson (1976). Solid lines connect mean data points.

preferred temperature range similar to the California legless lizards, is close to the predicted line. Of these four lizard species only the collared lizard shows a decreased Q_{10} at temperatures above its preferred temperature of 37°C. The lines for the California legless lizard and the golden sand skink are both close to the predicted line at their preferred temperature ranges and drop below the predicted line, with decreased Q_{10}s, above their preferred temperature ranges. The lines for the yucca night lizard (*Xantusia vigilis*) and the *Typhlosaurus lineatus* fall markedly below the predicted line at all temperatures studied.

Determination of the proximate causes of these metabolic patterns must await further study. However, since the reduced metabolic rates shown by the California legless lizard and the golden sand skink are apparent only above their preferred temperature ranges it might reasonably be suggested that the reductions could be due to altered enzyme activities at higher than optimum temperatures (see Hochachka and Somero, 1973; Hazel and Prosser, 1974).

Several hypotheses have been suggested to account for selective pressures for reduced metabolism at temperatures above the preferred range. One advantage of reduced metabolism at temperatures relatively high for California legless lizards may be in a reduction of evaporative water loss. Snyder (1971) suggested this advantage for the Yucca night lizard. McNab (1979) suggested a similar advantage for heteromyid rodents which have metabolic rates below those of other rodents. However, even at soil moisture contents as low as 0.65% water per unit mass of dry sand, the interstitial relative humidities measured for sand taken from the coastal dunes were greater than 90% (Fusari, 1982). Legless lizards would have to venture for long periods into very dry sand to gain much advantage in their respiratory water losses. It appears that they are generally reluctant to do so (Miller, 1944; Fusari, 1982). However, I have found *Anniella* on sunny days in the warm sand near the edges of leaf litter, where I have measured ground temperatures of 30°C or more and soil moisture contents below 0.5% at the very edges of the litter. I would expect that, under the rare but naturally occurring conditions, reduced respiratory water loss may aid in prolonging foraging time.

Mautz (1979a,b) related the reduced oxygen consumption, seen at all temperatures in several species of xantusiids, to a reduced energy need by small, sedentary lizards relative to larger, more active lizards. The California legless lizard is similar to the xantusiids in that it is small and secretive and does not appear to move about much (Miller, 1944; Bezy *et al.*, 1977). These ecological characteristics appear to fit *Typhlosaurus lineatus* as well and may be common in other species of legless sand swimmers (Huey and Pianka, 1982; Huey *et al.*, 1982). Therefore energy conservation may be another significant factor in the selective

pressure toward reduced metabolism at the energy-expensive high temperatures. Kamel and Gatten (1983) have found that the California legless lizard shows reduced active, aerobic metabolism as well as low active and inactive anaerobic metabolism at 20°C. They point out that, with a relatively normal metabolic scope, these lizards have the advantage of normal "metabolic expansibility" with a low maintenance cost.

An advantage for small, burrowing mammals, in having a reduced metabolism at relatively high ambient temperatures, is avoidance of a heat load (MacMillen and Lee 1970). Because the metabolic heat production of lizards is far below that of mammals it is unlikely that the very slight reduction seen in metabolism at high temperature could be important to these lizards in their natural environments. Under conditions of high interstitial relative humidities and at temperatures close to the critical thermal maximum (34°C, Brattstrom, 1965) it is possible that a reduced heat production might temporarily compensate for the reduced evaporative heat loss and allow a lizard time to escape to more favourable conditions.

It is important to remember that natural selection includes all relevant factors in the organism–environment interaction as adaptations evolve. If the reduced metabolism represents something more than a simple limitation of metabolic activity at temperatures above the preferred range, then I would expect that multiple factors (water conservation, energy conservation, thermal balance, etc.) were important in conferring adaptive value to the reduced metabolism of the California legless lizard. However, since this lizard can easily avoid either water or thermal stress by moving about within its habitat, the argument for energy conservation seems most convincing as the predominant advantage of the reduced metabolism under average, daily conditions. The other two factors would be advantageous under occasional, extreme conditions.

In summary it appears that the pattern of aerobic metabolism of the California legless lizard was related to its ecological niche, as a fossorial lizard of relatively mesic habitat. The physical demands of the fossorial environment would have acted as selective pressures on the metabolism as they appear to have guided the ecological patterns of fossorial vertebrates (Bezy and Wright, 1971).

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SEASONAL VARIATION IN SIZE AND FUNCTION OF THE NASAL SALT GLAND OF THE FRANKLIN'S GULL (*LARUS PIPIXCAN*)

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Abstract—1. Extrarenal salt excretion through the nasal gland allows marine birds to drink salt water. Species inhabiting fresh water usually have small glands, although in many species studied, the glands increase in size in response to salt loads.

2. The Franklin's gull (*Larus pipixcan*) breeds in freshwater habitats and winters in marine habitats. Accordingly it shows an annual cycle in the size and function of the nasal gland. We found a large and active gland in gulls collected in April (just after arrival on the breeding ground) and in October (just prior to migration), while birds collected from late May to July had small and apparently inactive glands.

3. Recrudescence of the gland occurred in wild-caught adults maintained in captivity on fresh water without a salt load.

4. This appears to represent a cyclical phenomenon that is independent of actual salt loading.

5. The glandular hypertrophy produced a histological picture similar to that of adaptive hypertrophy in response to salt loading.

6. The increase was not inhibited by maintaining birds on a constant 16 : 8 light/dark cycle. This change is analagous to size changes in avian gonads.

7. This cyclical phenomena may preadapt the Franklin's gull's salt gland to handle the slat load it will encounter when it reaches its winter quarters on the Pacific ocean shore.

INTRODUCTION

The nasal gland is present in all birds, although it is well-developed mainly in marine species (Ingue, 1963). The presence of the gland has been known for many years, but its excretory function was recognized relatively recently (Schmidt-Nielsen *et al.*, 1958). Technau (1936) recognized the size difference between the nasal glands of marine and non-marine birds, and Staaland (1967) summarized the diversity in size, morphology and function for the Charadriiformes. In all marine birds tested thus far, the nasal glands serve as functional salt glands capable of excreting sodium chloride in amounts up to 10% of the bird's body weight within 10 hr of salt ingestion (Cooch, 1964). In other words, the gland secretion can reach a sodium concentration approximately twice that of seawater (Schmidt-Neilsen and Fange, 1958; Fange *et al.*, 1958).

In most marine birds the glands are located supraorbitally, lying in a depression atop the bony rim of the orbit. Studies of salt secretion by the gland have been performed mainly on marine birds that are normally exposed to salt throughout the year and on domestic waterfowl (Peaker and Linzell, 1975). Natural seasonal changes in the size and activity of the glands have not been reported although many migratory species of birds are faced with a salt load for only part of the year. Accordingly we tested the hypothesis that in such species the salt gland would undergo an increase in size and function at a time when the species would likely be faced with a salt load, and that such a change would be independent of dietary sodium.

This hypothesis was tested on the Franklin's gull (*Larus pipixcan*), which breeds in freshwater marshes in the northern prairie regions of North America, and winters primarily off the west coast of South America. The species requires a functional salt gland during the winter, but presumably not during the breeding season. This paper describes temporal changes in size and histology of the Franklin's gull salt gland in wild birds collected from April through October, and describes the results on experimental gulls maintained in captivity on fresh water and on a diet without excess sodium.

MATERIALS AND METHODS

Franklin's gulls arrive at their Minnesota breeding grounds in April, begin nesting in May, have fledged young in July, leave the colony for nearby feeding areas by the end of July, and depart for their coastal wintering grounds by late October (Burger, 1974). They thus spend slightly more than half of the year in inland freshwater habitats.

Ten adult Franklin's gulls were collected at two week intervals from 24 April to 20 June and again on 23 July, at their breeding colony on the Agassiz National Wildlife Refuge, Marshall County, Minnesota. These birds were adults in breeding condition with brood patches. In addition, five adult and five juveniles (*ca* 5 months of age) were collected on 20 October, 450 km southeast of the refuge. These birds were part of a large aggregation of gulls which included some adults and young that had previously been color marked at the Agassiz breeding colony (Burger, 1972).

Birds were weighed, dissected, sexed by examination of the gonads, and the heads were preserved in 6% formalin. The left

nasal gland was removed from each gull and replaced in formalin for an additional three days. Each gland was then blotted dry with an absorbent towel, and its wet weight was determined immediately to 0.1 mg on an analytical balance. Glands were prepared for microscopic examination with haematoxylin and eosin and trichrome stains.

The degree of vascularity was estimated by counting the number of erythrocytes per ten randomly selected oil immersion fields. Following Peaker and Linzell (1975) the activity and degree of hypertrophy of the gland were estimated from the height of the secretory epithelial cells in the periductal area. The radius of the central lobe of each bird was determined from the average of six transverse sections randomly selected from the middle third of the gland. The major and minor axes were summed and divided by 4 to give a radius for comparability with Staaland (1967). The radius of the lobe increases both due to cellular hypertrophy and to hyperplasia. The statistical procedures employed were one-way and two-way analyses of variance on log-transformed data.

Thirteen adult Franklin's gulls captured at the colony on 23 July were maintained in captivity until October when they were sacrificed. They were fed on commercial dog food (sodium content 12.2 mg sodium/g dry wt). The sodium content was not significantly different from that of earthworms (10.2 mg/g) or insect larva, (9.2 mg/g, Edwards *et al.*, 1970), their natural foods. These birds were maintained on *ad libitum* fresh water. Seven of the birds were maintained at the ambient day : night schedule while six were maintained on a 16 : 8 hr fixed light : dark cycle.

RESULTS

Gross morphology

The supraorbital salt gland of the Franklin's gull differs morphologically from that of the Herring gull (*Larus argentatus*) in a significant characteristic. There are usually 5 (range 4–6) lobes in each gland and these are arranged linearly in a sheet (Fig. 1), an arrangement typical of species such as the Wood sandpiper (*Tringa glareola*) which similarly breeds in freshwater and migrates to marine habitats. By contrast the Herring gull, which over most of its range is a coastal species throughout the year, shows 20 or more lobes in at least two layers, a pattern characteristic of marine species (see Staaland, 1967).

In the Franklin's gulls the dorsal surface of the gland measured about 17 mm long and 6 mm wide (from edge of orbit to midline of the skull). The depth of the hypertrophied active gland is about 1.0 mm, and the weight (of a single gland) is about 120–150 mg. The depth and weight of the gland in the regressed, inactive state is about 0.6 mm and 50–80 mg.

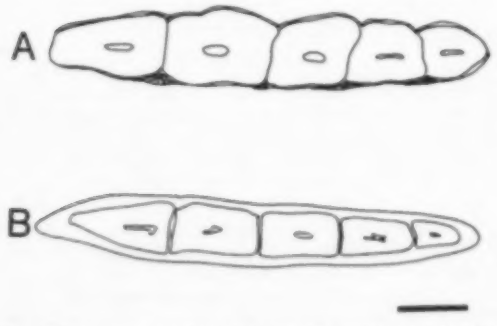

Fig. 1. Transverse sections through the nasal salt gland of Franklin's gull showing the active secretory gland (A) and the inactive gland (B). Note the distended capsule of the former and the shrunken state with prominent capsule in the latter. Stippled area on ventral surface of A represents blood vessels. Scale = 1 mm.

Gross examination revealed that in April and October, the glands of all birds abutted at the medial line of the frontal bone. The dorsal surface of the gland was convoluted (Fig. 2A) and the ventral surfaces were highly vascularized. By contrast birds collected in June and July showed smaller glands which did not meet at the midline (Fig. 2B), and were smooth rather than convoluted, with little apparent vascularity. Statistical analysis (Anova) of the gland weight showed a difference between April and October samples (Anova $P < 0.05$), but not among May, June and July samples. There was a highly significant difference in weight ($P < 0.01$) for the May/June/July vs April/October samples (Fig. 3).

The mean weight of the salt gland (Fig. 3) was high in April, decreased thereafter until July, and increased to a "peak" weight by October, after which time the birds could no longer be found in Minnesota. The ratio of gland weight to total body weight was examined seasonally (Fig. 3, top) and compared to data summarized by Peaker and Linzell (1975).

Histological examination

June/July specimens (Fig. 4B) show a disproportionate prominence of connective tissue. The central ducts are small and irregular in outline. The glandular tissue is shrunken away from the connective stroma, both between lobes, and under the gland capsule. The vascularity of the glands is reduced and there are few red blood cells in each field. The periductal area is occupied mainly by connective tissue. The secretory tubules are relatively short in length, and the cells themselves are shrunken in height.

October specimens (Fig. 4A) show a narrow capsule bulging and distended by the increased volume and number of secretory cells. This produces the glandular outline seen in Figs. 1 and 2(A). The central duct is distended and regular in outline. There are more red blood cells per field. The individual lobes are pressed together rather than distinct. There is disproportionate increase with the rear (caudad) end of the gland nearly twice the diameter of the front end. There is no evidence of edema or intracellular water storage. The glandular cells are in a secretory state, with a greater cell height, and the secretory tubules are lengthened, thereby increasing the average radius of each lobe.

The mean sizes of the lobes were calculated for six July and six October birds (see Table 1). We computed the average radius (length + breadth divided by 4), and the average area of each lobe. The average radius changed from 0.42 mm in July to 0.56 mm in October (Anova, $P < 0.005$). The average lobe area increased from 0.55 to 0.98 mm^2 (Anova, $P < 0.001$). The mean height of cells along secretory tubules in the central lobe of gland increased from 10.0 to 15.9 μm ($P < 0.001$) between July and October. A crude estimate of the increased vascularity of the gland was obtained from the number of erythrocytes per oil immersion field which increased from 7.8 to 25.2 ($P < 0.025$).

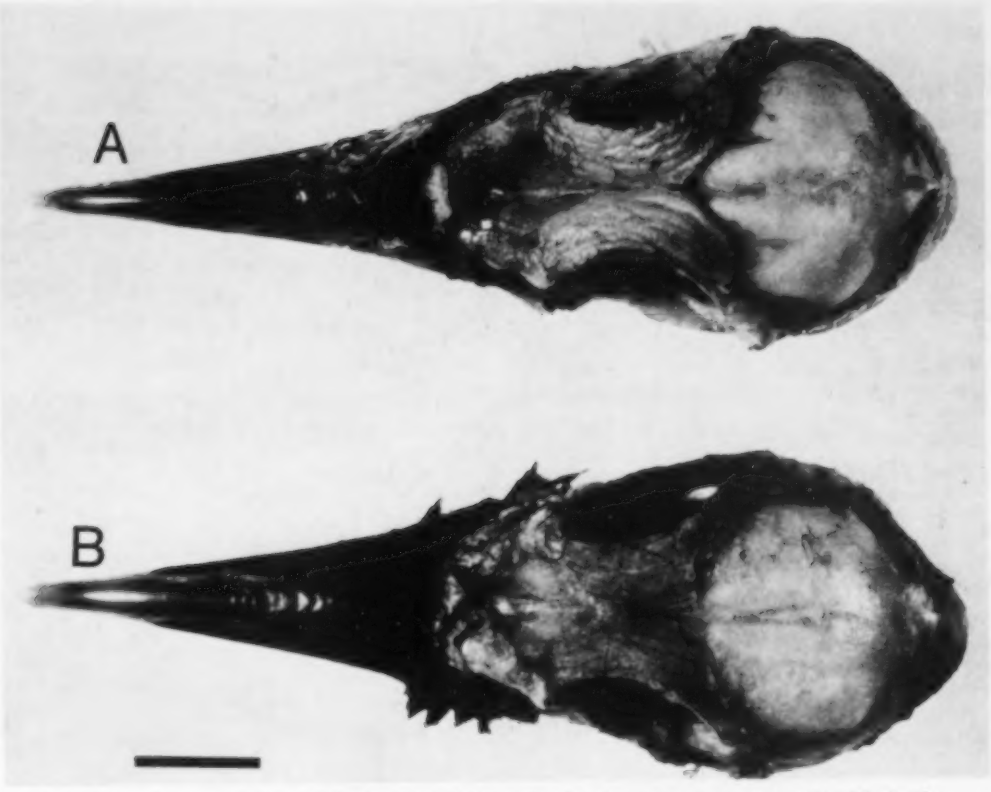

Fig. 2. Dorsal view of dissected heads of Franklin's gulls, showing an April specimen (A) with glands meeting at the midline and with a wrinkled dorsal surface, compared with a July specimen (B) showing the glands in a shrunken and flattened state. Scale = 1 cm.

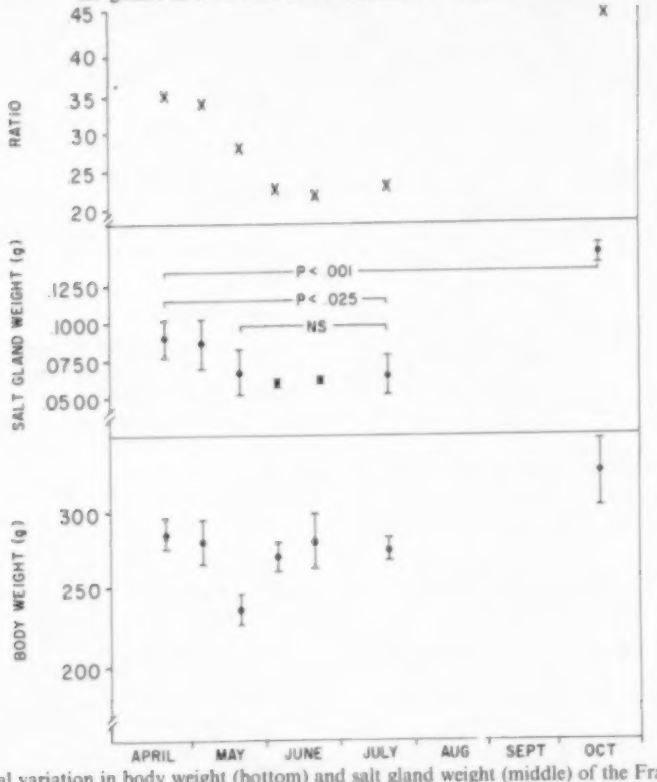

Fig. 3. Seasonal variation in body weight (bottom) and salt gland weight (middle) of the Franklin's gull. Shown are mean (black dots) ± 2 SD. Horizontal lines (middle panel) provide statistical comparisons among samples from different months. The seasonal change in ratio of mean gland weight to mean body weight (top) shows that the gland change is independent of the body weight.

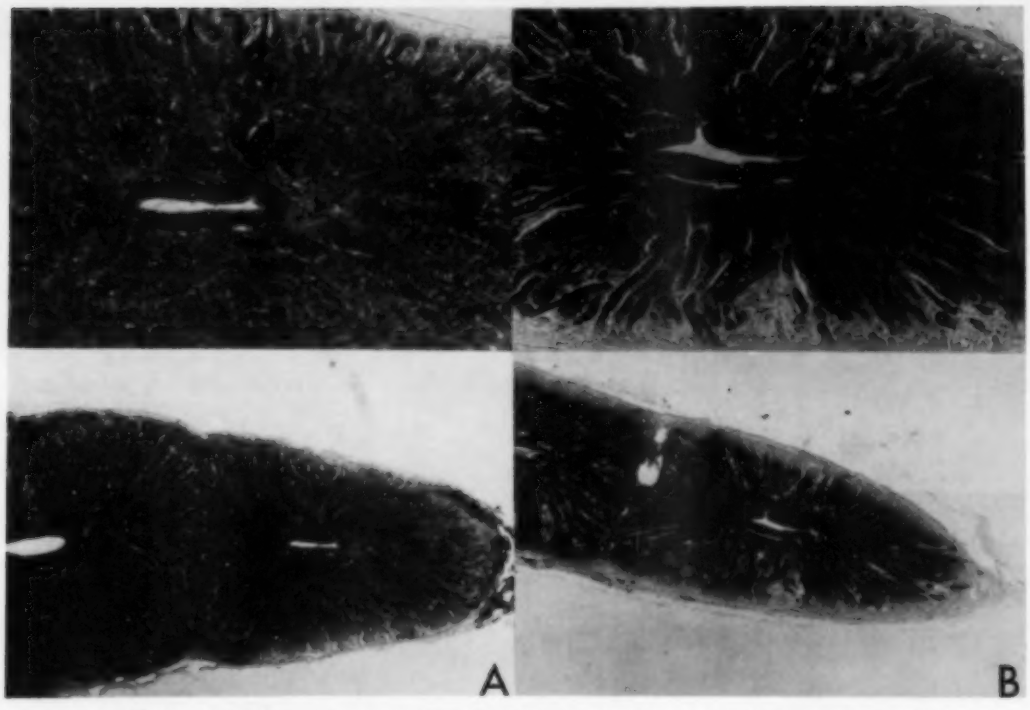

Fig. 4. Histological specimens from an active gland (A) and an inactive gland (B). Hematoxylin and eosin stains.

Experimental birds

The salt glands of the 13 adult gulls captured in July and sacrificed in October, were significantly heavier (mean 0.135 ± 0.016 g) than glands from the wild birds in July (mean 0.063 ± 0.015 g; Anova, $P < 0.001$). However, there was no significant difference between the captive birds and the wild birds collected in October ($P > 0.05$). Also there was no significant difference between glands of birds maintained on the normal decreasing vs fixed long day length (16 : 8) photoperiods ($P > 0.05$). Thus the lack of exposure to decreasing day length did not inhibit glandular recrudescence.

Histologic examination of the glands of the captive birds revealed that the lobe radius, cell heights, and average number of erythrocytes per oil immersion field fell within the lower portion of the range of the wild birds collected in October. These measures did not differ significantly from the October birds ($P > 0.05$).

Table 1. Morphometric comparisons of regressed and hypertrophied salt glands of Franklin's gulls

State of gland	($P <$)	July specimens Regressed	October specimens Hypertrophied
Average radius of lobes (mm)	0.005	0.42 ± 0.01	0.56 ± 0.08
Average area of lobes (mm^2)	0.0001	0.55 ± 0.03	0.98 ± 0.05
Cell height in Periductal area (μm)	0.001	10.1 ± 2.6	15.9 ± 2.5
Erythrocytes per oil immersion field	0.025	7.8 ± 2.2	25.3 ± 8.9

DISCUSSION

Three aspects of this study warrant discussion: (1) the histological changes in the gland; (2) the adaptive significance of size increase in birds maintained on freshwater and a solid diet without excessive sodium chloride and (3) a morphological comparison of Franklin's gull salt glands with those of other Charadriiformes.

Histological changes

Ballantyne and Wood (1967) showed that although salt gland secretory cells maintained a normal frequency distribution of cell diameter, the mean shifted from 9.7 to 11.9 μm with salt loading. Komnick and Kniprath (1970) found that whereas for peripheral cells the radius of cell height to tubule radius did not differ in salt and fresh water adapted Herring gulls, there was a significant difference for central cell height. At the same time the diameter of the central portion of the tubule was also much increased and the ratio of the two measures remained nearly constant.

The arrangement of the lobes of the Franklin's gull is characteristic of freshwater/saltwater species rather than of such coastal species as the Herring gull. Interestingly, Staaland's (1967) graph shows that Black-headed gulls (*L. ridibundus*), a species more similar in many respects to Franklin's than to Herring gull, is intermediate between the two species in average gland weight and in number of lobes (nine). Although Staaland (1967) demonstrated a more or less linear relationship between gland weight and number of

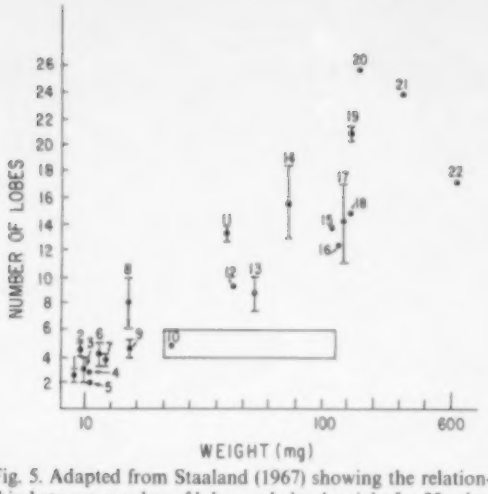

Fig. 5. Adapted from Staaland (1967) showing the relationship between number of lobes and gland weight for 22 other species. The Franklin's gull data obtained in this study are enclosed in the black rectangle which falls below the regression line for the other species. Numbers are for the following species: (1) *Tringa ochropus*; (2) *Actitis hypoleucus*, chicks; (3) *T. glareola*; (4) *Calidris minuta*; (5) *Gallinago gallinago*; (6) *Actitis hypoleucus*, adult; (7) *Charadrius hiaticula*; (8) *C. maritima*; (9) *Pluvialis apricara*; (10) *C. alba*; (11) *Limosa limosa*; (12) *C. canutus*; (13) *Larus ridibundus*; (14) *Alle alle*; (15) *Rissa tridactyla*; (16) *Alca torda*; (17) *Cepphus grylle*; (18) *Fratercula arctica*; (19) *Uria aalge*; (20) *U. lomvia*; (21) *Larus argentatus*; (22) *L. hyperboreus*. Numbers 1–12 are shorebirds; 13, 15, 21 and 22 are gulls; 14, and 16–20 are alcidae.

lobes in 22 species (see Fig. 5), the Franklin's gull data fall below the regression line (see rectangle in Fig. 5).

Adaptive hypertrophy

The comprehensive monograph by Peaker and Linzell (1975) summarizes numerous experiments and examines the lines of reasoning related to both the proximal and ultimate factors involved in secretion by and adaptive hypertrophy of the salt gland. It has been recognized that there is a difference between between size and function of the salt glands in marine birds versus those living on fresh water, but most students have been impressed with the rapid increase in size of the gland (adaptive hypertrophy) when freshwater adapted birds are subject to salt load. Less well-known, but nonetheless impressive is the corresponding decrease in size when birds are returned to strictly fresh water intake (McArthur and Gorman, 1978), suggesting that maintenance of a large and potentially active gland is energetically costly.

Previous salt gland studies have examined the structure and function of active glands (e.g. Fange *et al.*, 1958; Schmidt-Nielsen, 1960), and have not considered annual rhythms of activity, imposed on these glands by changes in habitat and salt load. In typical studies birds were subject to osmotic stress which resulted in increased nasal salt secretion (e.g. Holmes *et al.*, 1961; Cade and Greenwalt, 1966). For example, salt-stressed Mallard Ducks (*Anas platyrhynchos*) showed adaptive hypertrophy of the salt gland, increasing dramatically the volume (but not the concentration) of their nasal gland secretion (Schmidt-Nielsen and Kim, 1964).

The mechanism of salt transport from the blood to the gland's lumen (Thesleff and Schmidt-Nielsen, 1962) and the contribution of the gland to water balance have been investigated (see for example Schmidt-Nielsen *et al.*, 1968; Peaker and Linzell, 1975). These studies have shown that an increased salt load stimulates gland activity. A species breeding in freshwater and migrating to salt water (3.5% salt content) would either have to maintain a large responsive gland throughout its breeding season, or would have evolved a mechanism for turning on salt gland activity immediately upon or preferably even before its arrival in the marine habitat.

The energetic costs of maintaining a large potentially active gland are unknown, but might be unacceptable during the breeding season when other energetic demands (egg-formation, parental care) are high. In fact, during incubation (late May), Franklin's gulls of both sexes lost more than 25% of their body weight (Burger, 1974). However, the recrudescence of such a gland in the immediate post-breeding period might compete energetically with other costly processes, molt and pre-migratory fattening or migration.

Therefore a mechanism providing for an annual cycle in salt gland activity would have a high selective value in protecting an individual from undue physiological stress when it first arrives in the marine environment. Investigation of a possible hormonal basis for salt gland recrudescence would therefore be important.

Body and salt gland weights in Franklin's gulls

It could be argued that after reaching a low point in body weight during the nesting cycle, Franklin's gulls are able to undergo premigratory fattening with a concomittant increase in the weight of various organs, including the salt gland. Figure 3 shows that this is not the case; the increases in body weight and gland weight are out of phase. The birds begin regaining body weight as early as June, while the gland increase was not apparent even by late July. Figure 6 provides data on a sample of birds, showing no correlation (Kendall tau; $P > 0.10$) between gland weight and body weight for either males or females.

Birds collected in April about two weeks after arrival near the breeding grounds (Burger, 1972) still had a moderately large salt gland (mean = 98 mg). The mean weight of the gland had decreased slightly by early May, and this sample showed the greatest variance—some birds showing complete regression by that time, perhaps reflecting different dates of departure from the marine environment, or different energetic investments in reproduction. The lowest gland weights were in June and July. Two way analysis of variance by date and sex revealed no significant sex differences with regard to either body weight or gland weight, while there was significant difference by date for both body weight ($P < 0.05$) and gland weight ($P < 0.01$). Cornelius (1982) compared salt gland weights in seven species of waterfowl (Anatidae), and found that in four species males had significantly heavier salt glands than females, but he had no data on body weights. We cannot interpret this possible sexual dimorphism, in view of the fact that across many species there is a tendency for larger birds to have larger salt glands, whereas our Franklin's gull data

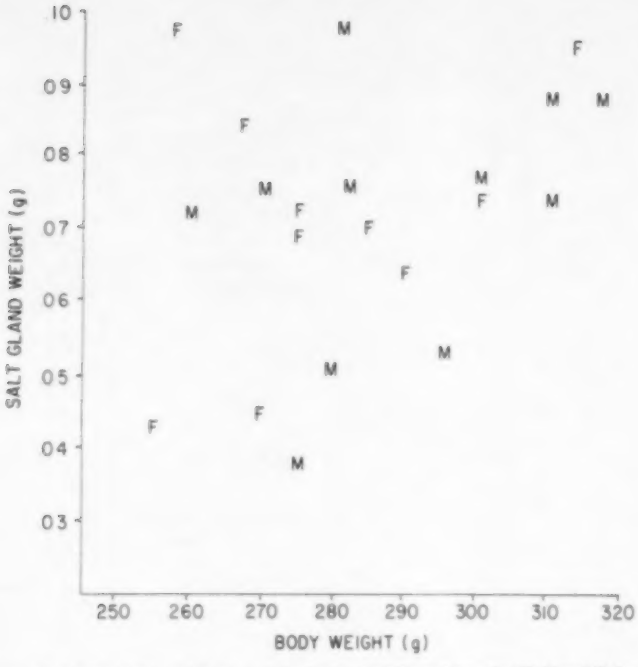

Fig. 6. Scattergram of gland weight and body weight for male (M) and female (F) Franklin's gulls, showing no direct relationship for either sex ($P > 0.10$), although the overall correlation is barely significant ($P < 0.05$).

showed no correlation between body weight and gland weight within a sample of birds collected in midsummer (glands inactive).

Although gulls collected in southern Minnesota in October had significantly heavier salt glands than the July sample, there is no evidence that in the wild, the gulls experience a higher salt load in October, than earlier in the breeding season. The foods consumed by the birds (primarily earthworms and insects) do not change during this period. Studies of dispersal of banded gulls did not reveal a tendency for movement westward into the area where lakes with high alkalinity and saline content predominate (Burger, 1972). The lakes in Minnesota range from 1 to 10 ppm chloride ion (Moyle, 1956).

Short term control of salt gland size and function: salinity

Numerous experiments, adequately summarized by Peaker and Linzell (1975) showed that within a few days after exposure to salt water (not necessarily seawater), birds of many species underwent an adaptive hypertrophy with increase in the size of the gland and the volume of secretion. Holmes and Stewart (1968) reported that ducklings underwent a doubling of gland weight within 5 days of salt exposure. The increase in salt gland size has two components, hypertrophy of existing cells and hyperplasia or production of new secretory cells at the periphery of the gland. Factors controlling this increase could include neural, osmotic, and hormonal, with evidence favoring the preeminence of the neural contribution (see discussion in Peaker and Linzell, 1975). It is important to distinguish factors initiating increased secretion and cellular hypertrophy, from factors promoting cell proliferation and glandular hyperplasia. These phenomena have different time courses ranging from minutes to hours to days (Peaker and Linzell, 1975), and we predict that hormonal factors will be important in initiating cellular proliferation at the periphery of the salt gland.

Very little work has been done on responses of salt glands in wild populations. Recently Cornelius (1982) examined salt glands of Redhead ducks (*Aythya americana*) collected by hunters on the wintering grounds along the Texas coast. He found that the gland weights were 2.5–4.5 × greater than the weights of glands from birds on the freshwater breeding grounds in Manitoba and Minnesota. This parallels our prediction that by the time the Franklin's gulls reach the coast their salt gland will have increased even further than the 150 mg weights found in Minnesota in October. Cornelius (1982) also found that even in Texas there was significant variation in mean gland weight as a function of habitat salinity. Moreover, his data showed a slight trend for increased gland weight from October through March, corresponding to continued residence time in the coastal estuaries. Thus it is apparent that the birds do show responses to ambient salinities. Our Franklin's gull data are very similar to the Redhead data, except that the October gulls had not yet migrated from their freshwater breeding grounds.

Long term control: photoperiod, annual cycles and hormonal influences

The prediction that the salt gland increase would occur in the absence of salt loading requires a mechanism that is independent of exogenous salt-loading. Data comparing the captive birds held until October

and a comparable sample of birds analyzed in July, revealed that the salt gland increased in weight (in captivity), even when birds drank only fresh water. There was no difference in birds maintained on a normal decreasing daylength vs birds maintained on a 16 : 8 hr light : dark cycle ($P > 0.05$; see Table 2).

Since the increase in salt gland weight in the wild birds taken in October and the captive birds maintained until October, seems independent of salt intake and photoperiod, it is proposed that the recrudescence of the glands might reflect either an endogenous rhythm (circennial of Farner and Follett, 1966), and/or some other exogenous stimulus (perhaps social) that predicts the environmental change. Although photoperiod seems the most likely exogenous stimulus, the initial experimental results do not support this. However, Farner (1964) and others concluded that in birds the photoperiodic control of the postnuptial molt, autumnal fattening and autumnal migration can all be indirect results of the increasing daylength of spring. That is, cyclic physiologic changes occurring in autumn after reproduction, are dependent on photoperiod changes many months earlier, and are not inhibited by abnormal photoperiod in the autumn. The captive birds would, therefore, have experienced a normal photoperiod at the appropriate time.

Prolactin, a hormone with widespread influence on salt–water balance in vertebrates was demonstrated to increase salt gland secretion (Peaker *et al.*, 1970). Ensor and Phillips (1970) found increased prolactin secretion within two days after subjecting ducks to 0.3 M sodium chloride, and they suggested that prolactin could be released from the pituitary upon arrival in a marine environment, thus facilitating adaptation to the marine environment.

Phillips and Ensor (1972) found that prolactin restored salt gland secretion in adenohypophysectomized birds. It seems reasonable that an annual cycle involving prolactin would facilitate the cyclical adaptive hypertrophy required by birds that breed in fresh water and migrate to a marine environment, as suggested by Peaker and Linzell (1975), but in our view the critical experiment would be to examine the effects of adenohypophysectomy and prolactin supplementation on birds restricted to salt water and on controls restricted to fresh water.

The developing salt gland may reflect another manifestation of fall migratory phenomena in the Franklin's gull and other species. The relationship of the size and function of the gland to endocrine factors and to other aspects of migration physiology, should provide a fruitful area for investigation.

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ABSORPTION OF NUTRIENTS FROM THE CORAL *POCILLOPORA DAMICORNIS* (L.) BY THE ECHINOID *EUCIDARIS THOUARSII* (Val.)

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Abstract—1. The absorption efficiency for *E. thouarsii* fed *P. damicornis* is 44.7% for carbohydrate, 85.3% for protein and 69.3% for lipid.

2. *E. thouarsii* fed *P. damicornis* absorbs 0.402 mg carbohydrate, 2.814 mg protein and 1.108 mg lipid for each g of *P. damicornis* ingested.

3. *E. thouarsii* fed *P. damicornis* absorbs 0.201 mg carbohydrate, 1.407 mg protein and 0.554 mg lipid per individual per day.

4. *E. thouarsii* fed *P. damicornis* absorbs 0.824×10^{-3} kcal carbohydrate, 7.950×10^{-3} kcal protein and 5.235×10^{-3} kcal lipid per individual per day.

5. Despite the high efficiency of digestive absorprion of organic constituents of the coral ingested, *E. thouarsii* must consume large quantities to meet its nutritional requirements.

INTRODUCTION

There have been numerous reports of the absorption efficiencies and utilization of plant foods by echinoids (Lawrence, 1975; Lawrence and Lane, 1982), but none have been done with animal foods. *Eucidaris thouarsii* grazes heavily on live coral in the Galapagos Islands (Glynn *et al.*, 1979). The purpose of this paper is to report the absorption efficiencies for the carbohydrate, protein, and lipid of *E. thouarsii* fed the coral *Pocillopora damicornis*, and to estimate the contribution which the absorbed organic material makes to the nutrition of the echinoid.

MATERIALS AND METHODS

Eucidaris thouarsii were collected from the Bay of Panama on 25 January 1982. Five individuals (41 ± 9 g wet wt, $\bar{x} \pm 1$ SD) each were placed in 5 one gallon jars supplied with running sea water and branches of *Pocillopora damicornis* on 27 January. Coral feces began to appear on 1 February and were collected daily from 5 to 12 February. Each day's collections of feces (coral fragments) from all jars were combined, drained of sea water, and then dried in a vacuum desiccator over concentrated sulfuric acid. Two samples of *P. damicornis*, each of 5 terminal branches, were drained of sea water and dried similarly. These were homogenized with a morter and pestle. Ash was estimated by combustion of the samples in a muffle furnace at 500°C for 4 hr (Paine, 1971). Protein was extracted in IN NaOH and estimated by the method of Lowey *et al.* (1951) with bovine serum albumen as the standard. Carbohydrate was extracted in hot 5% TCA and estimated by the phenol–sulfuric acid method of Dubois *et al.* (1956) with glycogen as the standard. Lipid was extracted in hot chloroform–methanol (2:1, v:v) and weighed by the method of Freeman *et al.* (1957).

Absorption efficiencies for the organic constituents were calculated by the indirect method of Lowe (Lowe and Lawrence, 1976):

$$U'_N = 100 \times \left(\frac{N'_f}{A'_f} - \frac{N'_e}{A'_e}\right) \Big/ \frac{N'_f}{A'_f},$$

where U'_N is the absorption efficiency for the organic constituent, N'_f and N'_e are the levels (percents) of the organic constituent in the food and feces respectively, and A'_f and A'_e are the levels of ash in the food and feces.

An absorption index was used to calculate the mg of each organic constituent ingested for each g dry weight of food ingested (Lowe and Lawrence, 1976): $A_N = (U'_N)(N'_f)$, where A_N is the absorption index, U'_N is the absorption efficiency for the organic constituent in the food, and N'_f is the level of the organic level in the dried food.

The absorption efficiencies were multiplied by the feeding rate of *Eucidaris thouarsii* on *Pocillopora* (0.5 g dry weight/individual per day) (Glynn *et al.*, 1979) to calculate the amount (g) of each organic constituent absorbed. The caloric equivalent of each organic constituent was calculated using the conversion factors of Brody (1945).

RESULTS

Pocillopora damicornis is primarily inorganic in composition (Table 1). Protein levels are higher than those of carbohydrate and lipid. While the level of ash in the feces of *Eucidaris thouarsii* is similar to that of the coral; the levels of protein, carbohydrate and lipid are much less (Table 1).

The absorption efficiencies for protein (85%) and lipid (69%) are high while that for carbohydrate (45%) is much less (Table 2). Much more protein than lipid is absorbed per unit of food eaten (Table 2) as a result of the higher level of protein in the coral and the higher

Table 1. Composition (in % dry weight) of *Pocillopora damicornis* and feces of *Eucidaris thouarsii* fed *P. damicornis*. The mean of two samples, each composed of five branches, is given for *P. damicornis*; the means ± 1 SD for samples collected for eight successive days are given for the feces

	Ash	Carbohydrate	Protein	Lipid
P. damicornis	96.2	0.09	0.33	0.16
Feces	96.5	0.05 ± 0.02	0.05 ± 0.03	0.05 ± 0.03

Table 2. Absorption efficiencies (in % of nutrient in food absorbed), absorption index (mg of organic constituent absorbed per g dry weight of coral ingested), and g and kilocalories organic constituent absorbed per individual per day for *Eucidaris thouarsii* fed *Pocillopora damicornis*

Constituent	Absorption efficiency	Absorption index	mg Absorbed/ individual per day	kcal Absorbed/ individual per day ($\times 10^{-3}$)
Carbohydrate	44.7	0.402	0.201	0.8
Protein	85.3	2.814	1.407	8.0
Lipid	69.3	1.108	0.554	5.2
Sum		4.324	2.162	14.0

efficiency with which it is absorbed. Very little carbohydrate is absorbed per unit of food eaten.

Lipid contributes 37% of the total amount of kilocalories absorbed while protein contributes 57% of the total (Table 2).

Carbohydrate contributes very little (6%) to the calories absorbed. The total number of kilocalories obtained by *E. thouarsii* fed *P. damicornis* is very small (14.0×10^{-3} kcal per day, Table 2).

DISCUSSION

Digestion and absorption reduces the already low levels of organic material in the coral to minimal levels in the feces, much less than those found for echinoid feces of plant origin (Lawrence, 1975; Lawrence and Lane, 1982). There are few studies of the absorption efficiencies of individual organic constituents of echinoids. The absorption efficiencies for carbohydrate, protein, and lipid of *Lytechinus variegatus* fed the alga *Halimeda incrassata* (Lowe and Lawrence, 1976), are almost identical to those found here for *Eucidaris thouarsii* fed *Pocillopora damicornis*. The absorption efficiencies of *L. variegatus* and *Strongylocentrotus purpuratus* fed various other marine plants (Lowe and Lawrence, 1976) were much less than those of *E. thouarsii* fed coral, except for *S. purpuratus* fed the alga *Macrocystis pyrifera*, for which the absorption efficiency for carbohydrate was high.

The low level of organic material in coral results in very low amounts absorbed by *Eucidaris thouarsii* per g of food ingested. This is particularly true with carbohydrate. More protein than lipid is absorbed. Values calculated for *Lytechinus variegatus* ranged from 26 to 83 mg carbohydrate absorbed (for five of six plant foods) and 9 to 21 mg lipid absorbed (for three of six plant foods) per g weight of food ingested; values calculated for other plants were even lower (Lowe and Lawrence, 1976). The amount of protein absorbed by *E. thouarsii* per g of coral ingested was 1×10^{-2} that absorbed by *L. variegatus* per g of *Halimeda incrassata* ingested, and about 4×10^{-3} that absorbed by *L. variegatus* per g of *Thalassia testudinum* ingested (Lowe and Lawrence, 1976).

It is obvious that the ability of *Eucidaris thouarsii* to obtain nutrients from *Pocillopora damicornis* is low. The absorption efficiencies for carbohydrate, protein, and lipid are moderately high. The fact that the modal size of the particles of *P. damicornis* ingested by *E. thouarsii* are relatively large (≥ 2 mm) may result in less efficient digestion than if finer particles could be browsed from the coral. Even if digestion and absorption of the organic constituents were complete, large quantities must be consumed to provide the nutritional needs of the echinoid. *Eucidaris thouarsii* either must consume more food or have fewer nutrient resources than echinoids feeding on plants or less calcified animals.

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AMMONIA PRODUCTION BY THE FRESHWATER BIVALVE *ELLIPTIO BUCKLEYI* (LEA): INTACT AND MONOVALVE PREPARATIONS

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Abstract—1. The production of ammonia, urea and ninhydrin-positive material was measured for clams with valves wedged open and with one valve removed (monovalve).

2. Accumulation of ammonia in the surrounding medium was linear with time in both intact and monovalve preparations. Ammonia production by intact animals was 0.194 μmol/g body wt/hr and by clams with one valve removed it was 0.136 μmol/g body wt/hr.

3. Urea was not detected and the production of ninhydrin-positive material was negligible.

INTRODUCTION

The catabolism of proteins and nucleic acids produces nitrogen-containing materials that may or may not be retained within an animal. Commonly these substances are superfluous or disadvantageous to the organism and are eliminated or rendered chemically innocuous. For aquatic invertebrates, a major product is ammonia which may be lost into the surrounding environment. However, other compounds such as urea, purines and free amino acids may also reflect this catabolism and be similarly eliminated or otherwise "set aside".

Ammonia production by the marine bivalves *Mytilus edulis* and *Mytilus californianus* has been reported to increase during periods when food sources were limiting or during gametogenesis. Both of these conditions might be expected to initiate an increased reliance on protein catabolism (Bayne and Scullard, 1977; Bayne and Widdows, 1978; Widdows, 1978). Similarly, Mann and Glomb (1978) concluded that increases in the amount of ammonia produced by *Tapes japonica* coincided with increased reproductive activity.

Alterations in ammonia production also occur during periods of osmotic stress. When the bivalves *Rangia cuneata*, *Modiolus demissus* and *Mytilus edulis* are exposed to hyposmotic conditions, the amount of ammonia excreted increases (Henry and Mangum, 1980; Bartberger and Pierce, 1976; Livingstone *et al.*, 1979). This loss of nitrogen is related to a decrease in the concentrations of intracellular free amino acids apparently functioning as osmolytes. Conversely, under hyperosmotic conditions the intracellular concentrations of these amino acids are expected to increase. At least a part of this increase is believed to be the result of ammonia fixation, excretory ammonia decreasing accordingly (Henry *et al.*, 1980; Livingstone *et al.*, 1979; Bishop, 1976).

It is thought that changes in the amount of ammonia produced reflect the degree to which nitrogenous compounds are being canalized or catabolized. Thus, measuring the accumulation of nitrogen-containing compounds produced by an animal can be useful in establishing "normal" patterns of elimination. Additionally, this catabolism can be experimentally altered so as to elucidate specific pathways. As a prelude to such work, it is necessary to establish baseline rates and qualitative patterns of catabolic nitrogen elimination. In this paper we report the development of an experimental system for evaluating these rates and patterns using the freshwater bivalve *Elliptio buckleyi* (formerly *Popenaias buckleyi* (Heard and Guckert, 1971)). Although studies on some major enzymes of nitrogen metabolism have already been undertaken on *Elliptio* (Rifkin, 1976; Falany and Friedl, 1981; Monahan, 1981), we are not aware of any comprehensive investigations on organismic nitrogen production by intact or semi-intact *Elliptio buckleyi*.

MATERIALS AND METHODS

Clams were collected from Lake Charles in Hillsborough County, Florida (82°28′30″N, 28°08′00″W). In the laboratory they were maintained unfed in aquaria containing water and substrate from the collection site. They were kept in this manner for at least one week before being used in experiments.

Prior to investigation, the valves of the clams were scrubbed to remove fouling organisms. The intact animals were then externally dried and weighed. At the end of an experiment, the soft parts of the animals were excised, blotted free of excess fluid on towelling and weighed.

In a typical experiment, the clams were placed in containers filled with a physiological salt solution (SS) buffered at pH 7.8 as the bathing medium. This solution was based on the "internal heart" solution for freshwater mussels described by Motley (1934) with phosphate buffer added. The medium was mixed in the containers using air-driven magnetic stirrers.

To ascertain if ammonia production varied proportionally with time, three clams with their valves wedged open were placed in containers with 30 ml of SS. Samples were removed hourly for ammonia analysis for a 6 hr period. Ninhydrin-positive materials and urea were also

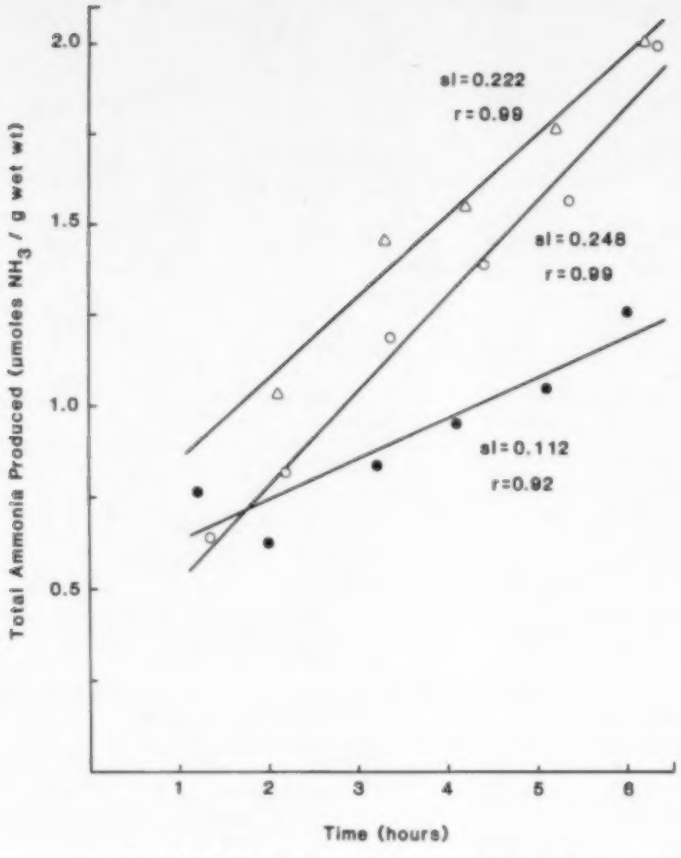

Fig. 1. Total ammonia produced by *Elliptio buckleyi* with valves wedged open. Data are shown for three clams. The rate of ammonia production is equal to the slope (sl) of the fitted line (r = correlation coefficient).

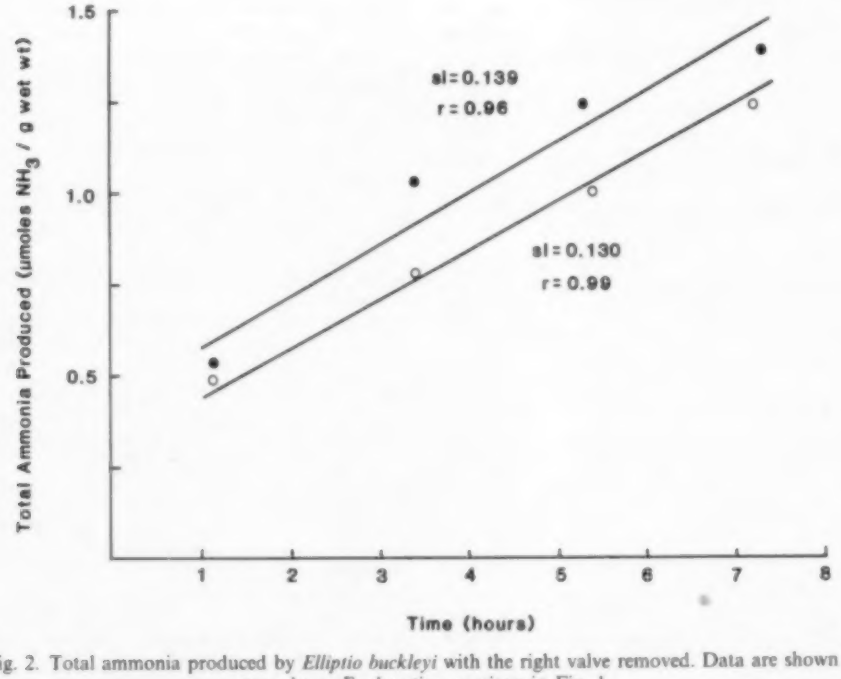

Fig. 2. Total ammonia produced by *Elliptio buckleyi* with the right valve removed. Data are shown for two clams. Explanation as given in Fig. 1.

measured in the samples taken at 3 hr and at the termination of the experiment.

The effect of the removal of one valve on the production of ammonia was investigated in a similar manner. For this experiment the right valve was removed by carefully cutting the corresponding muscle attachments, peeling down the mantle and breaking the hinge ligament. Preliminary work had indicated that immediately following the removal of a valve, there was measurable leakage of nitrogenous material, presumably from the wounds. Therefore, after this operation, six animals were maintained in SS for a 12 hr adjustment period. Subsequently they were transferred to experimental chambers and nitrogen production analyzed as above for 7 or 12 hr periods.

The phenol-hypochlorite method of Chaney and Marbach (1962) was used to measure ammonia. Substances reacting with ninhydrin were determined using a procedure outlined by Clark (1964). The amount of ninhydrin color due to ammonia alone was estimated using a pre-established correction curve. When this amount was subtracted from the absorbance of the sample, the remainder was considered due to "ninhydrin-positive" material. Urea was measured using 1-phenyl-1,2-propanedione-2-oxime as described by Archibald (1945). All assays were controlled with saline blanks and standards.

In all experiments, rates of ammonia production were calculated as slopes of lines fitted to the data using a least-squares regression (Zar, 1974).

RESULTS

Ammonia production into the surrounding medium proceeds at a mean rate ($\pm$SD) of 0.194 ± 0.072 μmol NH_3/g wet wt/hr when the valves of *E. buckleyi* are wedged open (Fig. 1). This rate was calculated from the slopes of lines fitted to the data ($r = 0.92$–0.99).

The time course of accumulation of ammonia into the medium surrounding monovalve preparations is shown for a representative experiment using two clams in Fig. 2 ($r = 0.96$–0.99). A visual comparison of these data with that for intact animals indicates that valve removal did not affect the pattern. The mean rate ($\pm$SD) of ammonia production by six clams with one valve removed, 0.136 ± 0.061 NH_3/g wet wt/hr is somewhat less, but nevertheless similar to the mean rate for intact animals. In all six cases, correlation coefficients were between 0.87 and 0.99.

Urea was not found in any of the experiments with intact or monovalve clams. Likewise, the accumulation of ninhydrin-positive material was negligible when corrected for the presence of ammonia.

DISCUSSION

Ammonia production by intact and monovalve preparations is comparable to that reported for other bivalves. *Tapes japonica* produces ammonia at a rate of 0.07–0.17 μmol NH_4^+/g live wt/hr (recalculated from Mann and Glomb, 1978), while a rate for *Mya arenaria* was found to be 5.5 μmol NH_3/g soft body parts/day (recalculated average from Allen and Garrett, 1971). Although some values reported for bivalves by Hammen (1968) are comparable to those found for *E. buckleyi*, ammonia production by some of the larger clams was considerably lower (i.e. 0.463 μmol NH_3/g tissue/day for *Mercenaria mercenaria*). It may be that ammonia production (and hence, elimination) bears a surface to volume relationship similar to that noted for respiration in animals (Bartberger and Pierce, 1976; Bayne and Widdows, 1978; Widdows, 1978; Mann, 1979).

That ammonia production from monovalve preparations was apparently not greatly altered by the removal of a valve is noteworthy. Such preparations can be more valuable in physiological and metabolic studies than the intact clam. In addition to allowing life processes of the animal to be easily monitored, the behavior of the soft parts can be observed and certain organs and tissues can be studied *in situ* by techniques such as ligation and perfusion.

It is not surprising that urea production by *Elliptio* was not found. When detected as an excretory product of bivalves, it has been present in small amounts. For example, Hammen (1968) reported only 0.082 μmol/g tissue wt/day for *Crassostrea virginica*. *Mya arenaria* produces only 3.5 μmol/day (recalculated from Allen and Garrett, 1971). If *E. buckleyi* did produce urea, it is possible it was not detectable over the time period studied.

As stated in the Introduction, ammonia need not be expected to constitute all of the nitrogenous waste production of aquatic invertebrates. Since the elimination of ninhydrin-positive material by monovalve preparations was negligible, it is likely that internal solutes were not lost in large amounts and that leakage due to the experimental treatment was minimal. It thus appears that the system was not grossly abnormal in respect to permeability and tissue leakage due to the trauma of valve removal.

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THE NATURE OF THE INCREASED SENSITIVITY TO INJECTED GTP OF THE SODIUM EFFLUX IN BARNACLE MUSCLE FIBERS PRE-EXPOSED TO ALDOSTERONE

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(*Received* 6 *April* 1983)

Abstract—1. A study has been made of the increased sensitivity to injected GTP of the sodium efflux in barnacle muscle fibers pre-exposed to aldosterone and of the problem whether or not aldosterone acts by raising the internal ATP level.

2. The results indicate that increased sensitivity to injected GTP develops fully some 8 hr following external application of 10^{-6}M aldosterone.

3. Neither actinomycin D nor cycloheximide abolishes this extrasensitivity. This is also true of colchicine and cytochalasin B.

4. The magnitude of the sustained response to injected "dialyzed" cholera toxin or cAMP-protein kinase catalytic subunit is practically the same as that of unexposed fibers.

5. Internal ATP levels in pre-exposed fibers are higher than in unexposed fibers, even in the presence of cycloheximide. Injection of ADP (0.1 M) raises the ATP levels and reduces the ArP levels, more so in unexposed fibers.

6. The suggestion is made that extrasensitivity of pre-exposed fibers to injected guanine nucleotides represents a post-translational phenomenon which might involve delay in the reassociation of R_2 with C (of cAMP-PK).

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INTRODUCTION

One long-standing problem in cell physiology is the need to understand the mechanism by which aldosterone is able to amplify the response of renal tubules to vasopressin (Schwartz and Kokko, 1980). In recent studies pursued in this laboratory, the subcellular site of action of aldosterone has been investigated by microinjecting GTP and its non-metabolizable analogue, Gpp(NH)p, into barnacle fibers that had been exposed overnight to the steroid *in vivo* (Bittar and Nwoga, 1981; Nwoga and Bittar, 1982). This particular work has revealed that the kinetics of the stimulatory response of the ouabain-insensitive Na efflux to injected Gpp(NH)p are identical to those seen with GTP, and hence they differ from those found in unexposed fibers in two notable ways. First, the magnitude of the stimulation in pre-exposed fibers is larger than that in unexposed fibers. And second, the stimulatory response in pre-exposed fibers is sustained rather than transitory. These striking differences in kinetics between pre-exposed and unexposed fibers have been explained by assuming that aldosterone acts at the level of the calmodulin-sensitive adenylate cyclase (Bittar and Nwoga, 1982a). However, to reach this new standpoint it has been necessary to address several relevant problems, chief among which were the following. (1) Is transcriptional or translational control the level at which aldosterone acts, or is it post-translational? (2) If the underlying mechanism turns out to be post-translational, is it then abolished by colchicine or cytochalasin B? (3) What is the role of internal ATP in the mechanism of sustained stimulation of the ouabain-insensitive Na efflux? This third question is of obvious importance, partly because aldosterone might act by increasing cell ATP, as suggested by Edelman *et al.* (1963), and partly because the effect of a raised internal ATP could be to suppress the reassociation of cAMP-protein kinase regulatory subunits (Rangel-Aldao and Rosen, 1977), thus leading to prolongation of the state of phosphorylation. As will be shown in this paper, neither transcriptional nor translational control is the level at which aldosterone acts. Rather what emerges is that one effect of the steroid in these fibers is to increase the levels of internal ATP. Whether aldosterone exerts an additional effect, that of recruiting the calmodulin form of membrane adenylate cyclase remains unclear. This is understandable since proof of such a hypothesis depends on the availability of a specific antagonist, e.g. a specific antibody and the demonstration that barnacle fibers possess this form of calmodulin. A preliminary account relating to portions of this work has already appeared (Bittar and Nwoga, 1982a).

MATERIALS AND METHODS

Materials. Specimens of *Balanus nubilus* were obtained from David King, Friday Harbor, Seattle, Washington and maintained in an Instant Ocean aquarium containing artificial sea-water. The temperature of the water was kept at about 12°C. Its composition was roughly as follows (mM): Na, 465; K, 10; Ca, 10; Mg, 60 (plus trace elements).

The procedure of overnight pre-exposure of a barnacle specimen to aldosterone consisted of injecting 1 ml of 10^{-6}M aldosterone solution into the cavity of the operculum, followed by suspending the barnacle in ASW containing 10^{-6} M aldosterone for a period of at least 8 hr.

Dissection, cannulation and microinjection. The dissection and cannulation of single muscle fibers, as well as the microinjection procedure were essentially the same as those described previously (Bittar *et al.*, 1972) except that the injector used was of the type described by Bittar and Tallitsch (1975).

Solutions. The bathing medium used was artificial sea water (ASW) of the following composition (mM): NaCl, 465; KCl, 10; $CaCl_2$, 10; $MgCl_2$, 10; $NaHCO_3$, 10 and pH 7.8. All experiments were carried out at a temperature of 23 ± 1°C.

Radiosodium measurements. These were carried out as described by Bittar *et al.* (1972). The flux data was processed and plotted as a fractional rate constant for ^{22}Na efflux. Changes in the magnitude of the Na efflux were estimated on the basis of these plots and expressed as a percentage change (mean ± SE of mean) of the efflux. Significance levels were estimated by using Student's unpaired *t*-test.

Internal ATP and ArP measurements. Total internal ATP in single fibers was measured using the firefly method (Bittar and Keh, 1980; Bittar *et al.*, 1983) and enzymic fluorimetry (Lowry and Passonneau, 1972). ArP was assayed by enzymic fluorimetry using arginine kinase. Briefly, single muscle fibers that had been frozen in liquid nitrogen, were homogenized using 0.1 N HCl in methanol. Before assay the pH of the supernatant samples was adjusted with $NaHCO_3$ to pH 7.5.

Infrafiber fluid volume was calculated as the difference between total fiber water (obtained by oven-drying) and extrafiber water which was taken as 5% of total fiber water.

cAMP-protein kinase type 2 catalytic and regulatory subunits. Both the catalytic and regulatory subunits of cAMP-dependent protein kinase type 2 were prepared from bovine heart and purified to homogeneity in Dr. Edmond Fischer's laboratory, as described by Bechtel *et al.* (1977) and Peters *et al.* (1977). cAMP was removed from regulatory subunit (R_2cAMP_4) by elution with 8 M urea after a brief period of equilibration followed by dialysis. The sample of R_{II} subunits free of cAMP was kept in 50% glycerol containing 4 mM MOPS, 100 mM KCl, 0.2 mM EDTA and 1 mM mercaptoethanol at pH 7.0. The sample of catalytic subunits was in 50% glycerol containing 30 mM KH_2PO_4, 150 mM KCl, 0.1 mM dithiothreitol and 0.1 mM EDTA at pH 6.8. The samples were dialyzed (before use) against 3 mM HEPES in water, pH 7.2 in the cold room and subsequently concentrated by applying Sephadex G-10 to the dialysis bag.

Agents. Ouabain, GTP, actinomycin D, cycloheximide, cytochalasin B and EDTA were purchased from Sigma Chemical Company, St. Louis, Missouri. 5'-Guanylylimidodiphosphate sodium was obtained from P. L. Biochemicals Inc., Milwaukee, Wisconsin. Mepacrine was from Pfaltz & Bower, Stanford, Conneticut and forskolin from Calbiochem-Behring, San Diego, California. Cholera enterotoxin was from Schwartz/Mann, Orangeburg, New York. When reconstituted with water to 0.1 ml, the solution contained 10^{-4}M cholera toxin, 0.5 M TRIS, 0.01 M $EDTANa_2$, 0.03 M NaN_3 and 2 M NaCl, pH 7.5. Imipramine HCl was a gift from Smith, Kline & French Laboratories, Philadelphia, Pennsylvania. Rabbit calmodulin antibody was a gift from Dr. L. J. van Eldik of the Howard Hughes Institute at Vanderbilt University, Nashville, Tennessee. This was dialyzed shortly before use against 3 mM HEPES in the cold room and then concentrated by applying Sephadex G-10 to the dialysis bag. Protein kinase inhibitor (PKI) was a gift from Dr. E. H. Fischer of the University of Washington, Seattle, Washington.

RESULTS

Sensitivity to injected GTP of fibers pre-exposed in vitro *to aldosterone in varying concentration*

In this type of experiment, fibers from the same barnacle specimen were suspended in ASW containing aldosterone in varying concentration for a period of about 8 hr, and then 30 min before the injection of 0.5 M GTP, 10^{-4}M ouabain was applied externally. The results show that the magnitude of the stimulatory response to GTP was as follows: using 10^{-12}M aldosterone: $352 \pm 18\%$, $n = 3$; 10^{-10}M aldosterone, $508 \pm 22\%$, $n = 3$; 10^{-8} M aldosterone, $433 \pm 27\%$, $n = 3$; and 10^{-6} M aldosterone, $451 \pm 11\%$, $n = 3$. These values including that obtained with 10^{-12}M aldosterone are significantly greater than the control value of $284 \pm 10\%$ observed with unexposed fibers ($n = 3$). It therefore seems safe to say that increased sensitivity to GTP can be induced by aldosterone under *in vitro* conditions, even with steroid concentrations that fall within the physiological range.

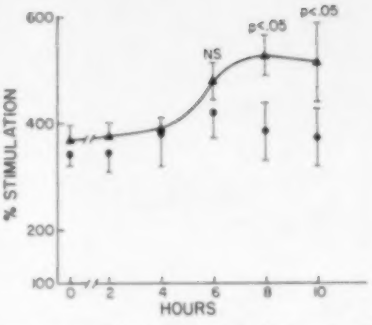

Fig. 1. Time-course of the development of full extra-sensitivity of fibers pre-exposed to 10^{-6}M aldosterone *in vitro* to the injection of 0.5 M GTP. Ordinate: % stimulation of the ouabain-insensitive Na efflux. Abscissa: hr. Vertical bars indicate SEM. Each point is the mean value of three measurements. The fibers used (▲, pre-exposed and ●, unexposed) were isolated from the same barnacle specimen.

Time-course of increased sensitivity to GTP

In order to draw a valid comparison between pre-exposed and unexposed fibers, a large number of fibers were isolated from a barnacle specimen and then exposed *in vitro* to 10^{-6}M aldosterone for varying periods of time. Another group of fibers served as control. Ouabain (10^{-4}M) was then applied externally 30 min prior to the injection of 0.5 M GTP. Summarized in Fig. 1 are the results of these experiments. This indicates a statistically significant rise in sensitivity to injected GTP 8 hr following the addition of aldosterone. A period of 8 hr is comparable to that required for increased sensitivity to develop *in vivo* (E. E. Bittar and J. Nwoga, unpublished observation) and is half that allowed in standard experiments involving pre-exposure overnight of the barnacle specimen to aldosterone. An additional feature which is noteworthy is that the magnitude of the responses estimated at $t = 8$ and 10 hr is practically the same as that observed in fibers following exposure *in vivo* to aldosterone.

Lack of effect of actinomycin D and cycloheximide

To determine whether augmentation of the response to injected GTP by aldosterone involves transcriptional or translational control, experiments were done with actinomycin D (an inhibitor of RNA synthesis) and cycloheximide (an inhibitor of protein synthesis). In the first group of experiments, a barnacle specimen was pre-exposed *in vivo* to 0.5 μg/ml actinomycin D in addition to 10^{-6}M aldosterone while another specimen was pre-exposed only to 10^{-6}M aldosterone. Some 16 hr later, fibers were isolated and treated

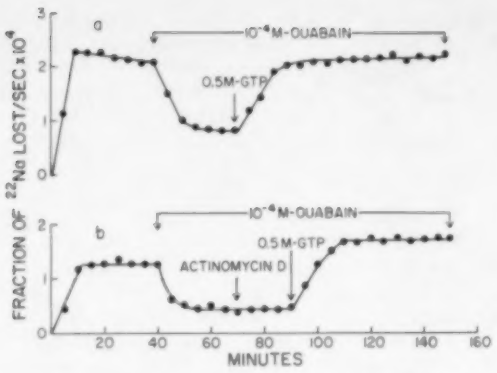

Fig. 2. (a) Kinetics of the response to injected 0.5 M GTP in a fiber pre-exposed overnight *in vivo* to 10^{-6}M aldosterone and 0.5 μg/ml actinomycin D. (b) Kinetics of the response to injected 0.5 M GTP in a fiber pre-exposed to 10^{-6}M aldosterone *in vivo* overnight, cannulated, treated externally with 10^{-4}M ouabain before injecting actinomycin D (40 μg/ml) and GTP in succession.

externally with 10^{-4}M ouabain prior to injection of 0.5 M GTP. As illustrated in Fig. 2a, the response to injected GTP is sustained and in the order of 442 ± 38% ($n = 3$), a value not different from that obtained with controls, *viz.* 400 ± 38%, $n = 3$, P being > 0.5. In parallel experiments, pre-exposed, ouabain-poisoned fibers were injected with actinomycin D (40 μg/ml) 20 min before the injection of 0.5 M GTP. Again, as illustrated in Fig. 2b, actinomycin D failed to modify the kinetics of the response to GTP (the size of the response being in the order of 389 ± 58%, $n = 3$ vs 397 ± 17% in controls, $n = 3$, P being > 0.5). It was therefore concluded that the mechanism of increased sensitivity to injected GTP does not depend on the synthesis of mRNA.

In the next group of experiments, a barnacle specimen was exposed *in vivo* to 10 μg/ml cycloheximide, as well as 10^{-6}M aldosterone, while a second specimen was exposed only to 10^{-6}M aldosterone. Some 18 hr later, fibres were isolated from both specimens and treated externally with 10^{-4}M ouabain before injecting 0.5 M GTP or 0.5 M Gpp(NH)p. The results obtained show a sustained response to GTP averaging 436 ± 47% ($n = 3$), compared with a sustained response averaging 471 ± 40% in controls, $n = 3$ (P > 0.4). Additionally the results show a sustained response to injected Gpp(NH)p averaging 524 ± 27%, ($n = 3$), compared with 510 ± 19% in controls, $n = 3$ (P > 0.5).

The above type of experiment was repeated but this time a barnacle specimen was exposed overnight to only 10^{-6}M aldosterone. Fibers from this specimen were then treated externally with 10^{-4}M ouabain and injected with 100 μg/ml cycloheximide, followed by 0.5 M GTP. Control fibers were poisoned with ouabain and injected with distilled water (0.4 μl) prior to the injection of GTP. The results show a sustained stimulation to GTP in test fibers in the order of 725 ± 102% ($n = 3$), compared with sustained stimulation in the order of 601 ± 60% in controls, $n = 3$ ($P > 0.2$). Thus, the conclusion drawn was that the increased sensitivity to injected guanine nucleotides found in pre-exposed fibers is not related to a mechanism at the level of translation.

Lack of effect of colchicine and cytochalisin B

Colchicine, a known microtubule disruptive agent and cytochalasin B, a disruptive agent of microfilaments (Borchers and Debrander, 1975) were used to eliminate the possibility of the role of these structures in the post-translational mechanism of action of aldosterone. In the first two groups of experiments, 10 μg/ml colchicine or cytochalasin B was applied externally to pre-exposed fibers shortly after the onset of peak stimulation by injected GTP (0.5 M). These agents were found to be ineffective ($n = 3$ in each group). In additional experiments, 10 μg/ml colchicine or cytochalasin B was applied externally to pre-exposed, ouabain-poisoned fibers prior to the injection of 0.5 M GTP. The results obtained show a sustained stimulatory response to GTP in the order of 662 ± 45% ($n = 3$), compared with 578 ± 22% in controls, $n = 3$ (P > 0.2) and a sustained stimulatory response to GTP in the order of 592 ± 121% ($n = 3$), compared with 578 ± 22% in controls, $n = 3$ ($P > 0.4$), respectively.

Response to a raised k_e

Earlier work led to evidence that the sensitivity of pre-exposed fibers to the injection of Ca^{2+} in graded concentration does not differ from that found in unexposed fibers (Nwoga and Bittar, 1983). Another way in which to approach this problem is suddenly to elevate K_e and hence reduce myoplasmic pCa by activating Ca^{2+} channels (Mason-Sharp and Bittar, 1981). The results obtained show that raising K_e from 10 to 100 mM resulted in stimulation of the ouabain-insensitive Na efflux in pre-exposed fibers in the order of 491 ± 65% ($n = 6$), compared with 467 ± 60% stimulation in controls, $n = 6$ ($P > 0.5$). This result seemed to rule out the possibility that the post-translational point of action of aldosterone is, for example, calmodulin/Ca-dependent protein kinase.

Response to imipramine before and after GTP

Keeping in mind that imipramine is an inhibitor of calmodulin (F. Siegel, private communication) and that this tricyclic antidepressant is able to almost completely reverse the sustained stimulation seen following the injection of Gpp(NH)p into pre-exposed, ouabain-poisoned fibers, experiments were done in which 5×10^{-5}M imipramine was applied externally to pre-exposed, ouabain-poisoned fibers prior to the injection of 0.5 M GTP. The results show a lack of effect of GTP injection ($n = 4$). In parallel experiments where 5×10^{-5}M imipramine was applied following the onset of peak stimulation by injected GTP (0.5 M), a marked reversal of the response was seen (89 ± 10% reversal of the step-up, $n = 8$). This particular result is illustrated in Fig. 3.

Response to GTP in the presence of mepacrine

Mepacrine, a known inhibitor of phospholipase A, completely inhibits the action of aldosterone on Na transport in the toad bladder (Yorio and Bentley, 1978). In view of this, and the fact that mepacrine is also an inhibitor of calmodulin (Volpi *et al.*, 1981), experiments were designed in which 10^{-4}M mepacrine was applied externally prior to the injection of 0.25 M

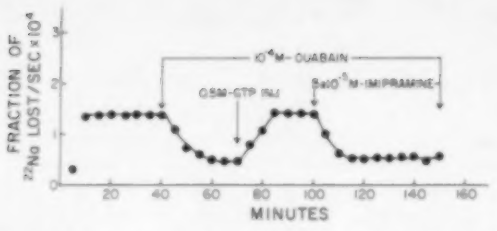

Fig. 3. Complete reversal of the response to injected 0.5 M GTP by external application of 5×10^{-5}M imipramine.

GTP. The results show $155 \pm 18\%$ stimulation by GTP, $n = 3$ vs $343 \pm 62\%$ in controls, $n = 3$, $P < 0.05$.

Lack of effect with calmodulin antibody

Numerous experiments involving the injection of calmodulin antibody before or after GTP [and before or after Gpp(NH)p] led to negative results. Two reasons for the lack of effect can be given: one is that the concentration of antibody injected into these fibers was too low, and the other is that IgG may well be relatively immobile in the myoplasm.

Response to dialysed cholera toxin

The observation that pre-exposed, ouabain-poisoned fibers have the same sensitivity to injected cholera toxin as unexposed fibers (Nwoga and Bittar, 1983) was taken to mean that GTPase which is a component of the adenylate cyclase complex and the target of cholera toxin action (Van Heyningen, 1982) is not the site of action of aldosterone. However, since these experiments were based on the use of cholera toxin in an aqueous vehicle containing TRIS, EDTA, NaN_3 and NaCl, it was decided to dialyze a sample of the toxin and then use it for injection. The results of these experiments show that the injection of 5×10^{-5} M cholera toxin (dialyzed) into pre-exposed ouabain-poisoned fibers caused $243 \pm 59\%$ stimulation ($n = 3$), compared with $285 \pm 23\%$ stimulation in unexposed, ouabain-poisoned fibers (NS). Since cholera toxin is now thought to act by ADP-ribosylation (Vaughan and Moss, 1982), an argument can be made for holding that aldosterone does not influence this reaction.

Response to injected EDTA

Bittar and Nwoga (1982b) reported that the injection of EDTA into ouabain-poisoned fibers leads to a transitory stimulation which is largely prevented from occurring by preapplying verapamil. The results of experiments with pre-exposed fibers show that the injection of 0.25 M EDTA caused sustained stimulation of the ouabain-insensitive Na efflux in the order of $135 \pm 17\%$ ($n = 3$), compared with $144 \pm 14\%$ stimulation in unexposed fibers, $n = 7$ (NS). Injection into the three test fibers of 0.5 M $MgCl_2$ after peak stimulation by EDTA resulted in complete reversal of the response. Whereas these results with EDTA resemble those obtained with injected $ATPNa_2$ and 5'-App(NH)p (Nwoga and Bittar, 1983), they differ from those obtained with GTP [or Gpp(NH)p] in two respects. First, they fail to show a distinction between exposed and unexposed fibers. This is, of course, not surprising if increased presence of guanine nucleotide is a prerequisite. And second, the size of the response to injected 0.25 M EDTA is about half that of injected 0.25 M GTP. This may be partly or wholly due to the fact that EDTA removes not only the available free Mg^{2+} but also the extra-free Ca^{2+} which becomes available following activation of Ca^{2+} channels.

EDTA followed by cAMP

The kinetics of the response to injected cAMP in pre-exposed and unexposed fibers are alike (Nwoga and Bittar, 1983). This implies that aldosterone does not act here by slowing the phosphodiesterase (PDE) system or by modifying the behavior of the cAMP-protein kinase system, e.g. by reducing the K_m for ATP. However, experiments of this type are vulnerable to the criticism that the response to injected cAMP is suppressed by the high internal free Mg^{2+} known to be present in these fibers (Ashley and Ellory, 1972). To dispose of this criticism, experiments were carried out in which 0.25 M $EDTAK_2$ was injected prior to 10^{-3}M cAMP. The results show that injected EDTA caused sustained stimulation, while cAMP caused a transitory response. This result is a telling argument against the idea that sustained stimulation by injecting EDTA or guanine nucleotides is due to suppression of PDE by aldosterone.

Response to injected R_2cAMP_4 following GTP

Nwoga and Bittar (1983) found that the injection of pure cAMP-protein kinase type 2 regulatory subunits free of cAMP following peak stimulation by injected Gpp(NH)p causes a partial reversal of the response. Since such inhibition might be related to the ability of *R* to bind cAMP, this type of experiment was repeated using R_2cAMP_4. As illustrated in Fig. 4, injection of 10^{-5}M R_2cAMP_4 some 15 min following the onset of maximum stimulation by GTP caused a rapid step-down in the stimulated efflux (averaging $45 \pm 4\%$, $n = 4$). This result strengthens the conclusion that the mechanism underlying the response to GTP involves in part activation of cAMP-protein kinase by newly formed cAMP.

Response to injected cAMP-protein kinase catalytic subunits, followed by protein kinase inhibitor

If the response to GTP is largely due to activation by newly formed cAMP of cAMP-protein kinase, and if this is mediated by membrane protein phosphorylation, then the question to be asked is: does aldosterone act by increasing the number of available phosphate acceptor sites? One obvious way in which to explore this possibility is to inject pure catalytic subunits and see if the response in pre-exposed fibers is greater than that in unexposed fibers. The results

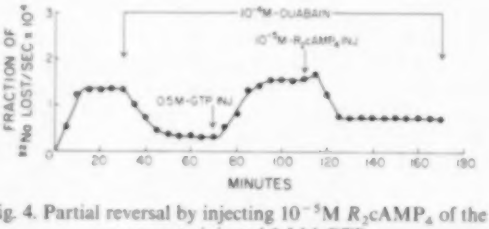

Fig. 4. Partial reversal by injecting 10^{-5}M R_2cAMP_4 of the response to injected 0.5 M GTP.

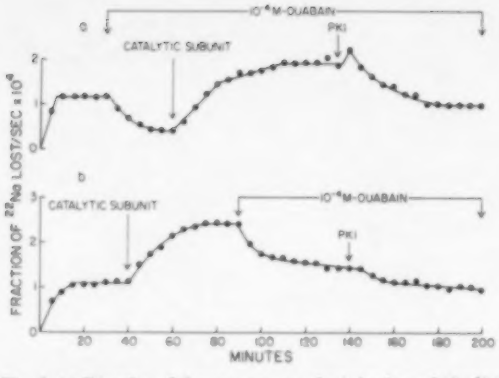

Fig. 5. (a) Kinetics of the response to the injection of 10^{-4}M catalytic subunit, followed by 10^{-4}M PKI in a fiber pretreated externally with 10^{-4}M ouabain. (b) Kinetics of the response to the injection of 10^{-4}M catalytic subunit, followed by 10^{-4}M ouabain externally, and then 10^{-4}M PKI internally.

obtained show that injection of 2×10^{-5}M catalytic subunit into pre-exposed, ouabain-poisoned fibers caused a sustained stimulatory response in the order of $314 \pm 27\%$ ($n = 6$). However, this value is not significantly different from $282 \pm 32\%$ sustained stimulation seen in unexposed, ouabain-poisoned fibers, $n = 19$. Because these experiments were important, they were repeated but this time ouabain was omitted. The results obtained show sustained stimulation following the injection of 2×10^{-5}M catalytic subunit into pre-exposed fibers in the order of $104 \pm 17\%$ ($n = 4$). This is not significantly different from $98 \pm 29\%$ sustained stimulation seen in unexposed fibers, $n = 6$. Illustrated in Fig. 5 are both types of experiments. Also shown is that the injection of 10^{-4}M protein kinase inhibitor (PKI) long after peak stimulation of the ouabain-insensitive Na efflux (upper panel) caused a prompt but partial reversal of the response (averaging $49 \pm 7\%$, $n = 6$). Shown in the lower panel is that injection of PKI long after external application of 10^{-4} M ouabain to a fiber injected with catalytic subunit beforehand resulted in a further decline in the residual efflux ($n = 4$).

Measurement of internal ATP in pre-exposed and unexposed fibers

To see whether there is a difference in total internal ATP between fibers pre-exposed overnight to 10^{-6}M aldosterone *in vivo* and unexposed fibers, three test and three control barnacle specimens were used. The values obtained for internal ATP by enzymic fluorimetry and the firefly method are given in Table 1, where it can be seen that both methods of assay give ATP values for pre-exposed fibers that are significantly greater than those found in unexposed fibers. In this connection, it is worth mentioning that the fluorimetric and firefly determinations were done using the supernatant samples of unexposed and pre-exposed fibers at about the same time and not on separate days.

Table 1. Total internal ATP and ArP (mmol/kg fiber water) contents of unexposed and pre-exposed fibers

	ATP (firefly)	ATP (fluorimetry)	ArP (fluorimetry)
Unexposed fibers	4.81 ± 0.38 $n = 15$	4.83 ± 0.21 $n = 18$	20.79 ± 0.89 $n = 18$
Pre-exposed fibers	6.47 ± 0.36 $n = 20$	5.70 ± 0.20 $n = 21$	19.69 ± 1.03 $n = 21$
P	<0.01	<0.01	>0.4

Lack of effect of cycloheximide on internal ATP and ArP levels

To answer the question whether the observed rise in internal ATP is related to a mechanism involving *de novo* synthesis of proteins, measurements of internal ATP (and ArP) were carried out on fibers from two barnacle specimens, one pre-exposed to 10^{-6}M aldosterone and 10 μg/ml cycloheximide, and the other only to 10^{-6}M aldosterone. As shown in Table 2, the levels of internal ATP as measured by enzymic fluorimetry and firefly, and ArP by fluorimetry, are not significantly different in the two groups of fibers. Although such data is in accord with the concept that aldosterone influences ATP levels as the result of an activation mechanism, the possibility remains that cycloheximide is itself able to raise the internal ATP level. To date, evidence in favor of this idea is unavailable (Bridger and Henderson, 1983).

Time-course of internal ATP and ArP levels in pre-exposed and unexposed fibers suspended in ASW containing 1 mM adenosine

There is evidence that the ATP content of cells, e.g. heart and skeletal muscle or liver cells (Seraydarian *et al.*, 1972; Lund *et al.*, 1975; Rapaport and Zamecnic, 1976) can be raised by simply adding adenosine externally. Shown in Fig. 6 is the time-course for the levels of internal ATP and ArP in unexposed and pre-exposed fibers suspended in ASW containing 10^{-3}M adenosine. Two features stand out. First, there is a

Table 2. Internal ATP and ArP contents (mmol/kg H_2O) of fibers pre-exposed to aldosterone and cycloheximide

Type of experiment	ATP (fluorimetry)	ATP (firefly)	ArP (fluorimetry)
Pre-exposed to aldosterone	5.39 ± 0.42 ($n = 4$)	6.73 ± 0.38 ($n = 4$)	18.46 ± 0.15 ($n = 4$)
Pre-exposed to aldosterone and cycloheximide	6.02 ± 0.36 ($n = 8$)	7.93 ± 0.45 ($n = 7$)	19.38 ± 1.04 ($n = 8$)
P	>0.3	>0.1	>0.5

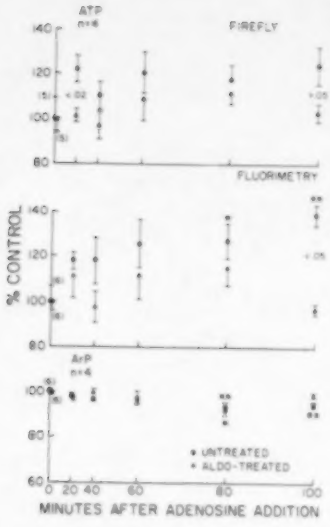

Fig. 6. Time-course of internal ATP and ArP levels in unexposed (●) and pre-exposed (▲) fibers suspended in 10^{-3}M adenosine-ASW. Upper panel: ATP values obtained with firefly. Middle panel: ATP values obtained with the fluorimetric method. Lower panel: ArP values obtained with the fluorimetric method. Unless stated otherwise in parentheses, the number of measurements done is that given in the left upper and lower corner of the panels. Vertical bars indicate SEM. Pre-exposed fibers were isolated from the same barnacle specimen, while unexposed fibers were isolated from another specimen belonging to the same batch of barnacles then available in the aquarium. * Denotes significant difference at $P < 0.05$. ** Denotes significant difference at $P < 0.02$.

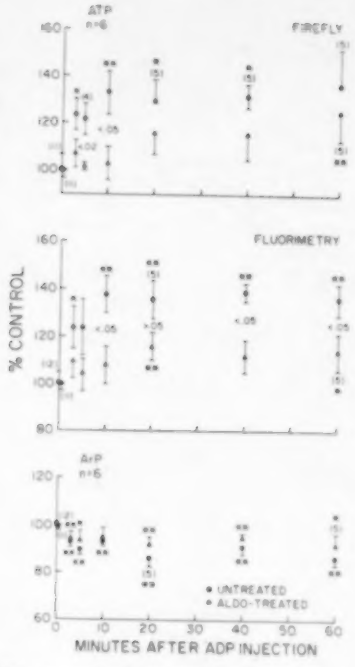

Fig. 7. Time-course of internal ATP and ArP levels in unexposed (●) and pre-exposed (▲) fibers suspended in ASW and injected with 0.1 M ADP at $t = 0$. Upper panel: ATP values obtained with firefly. Middle panel: ATP values obtained with the fluorimetric method. Lower panel: ArP values obtained with the fluorimetric method. Unless stated otherwise in parentheses, the number of measurements done is that given in the left upper and lower corners of the panels. Vertical bars indicate SEM. Pre-exposed fibers were isolated from the same barnacle specimen, while unexposed fibers were isolated from another specimen belonging to the same batch of barnacles then available in the aquarium. * Denotes significant difference at $P < 0.05$. ** Denotes significant difference at $P < 0.02$.

significant increase in the internal ATP level in unexposed fibers at $t = 80$ and 100 min on the basis of the fluorimetric method, and the difference between these values and those found in pre-exposed fibers at $t = 100$ min is significant, P being < 0.05. And second, the fall in ArP levels at $t = 80$ and 100 min parallels this rise in ATP. Such results are suggestive of adenosine incorporation into the ATP pool via the adenylate kinase reaction (i.e. adenosine→AMP, ADP→ATP) and acceleration of the forward arginine kinase reaction (i.e. ArP + ADP→Ar + ATP). The failure of ATP levels in pre-exposed fibers to rise significantly following the addition of 1 mM adenosine can be accounted for by supposing that ATP acts as a negative effector of phosphofructokinase and that it does so more potently when the enzyme is in the phosphorylated form and the internal ATP level is raised.

Time-course of ATP and ArP levels in unexposed and pre-exposed fibers injected with ATP

The idea that aldosterone reduces buffering against ADP accumulation was examined more closely by injecting 0.1 M ADP into unexposed and pre-exposed fibers. Shown in Fig. 7 is that the ATP levels in unexposed fibers undergo a fairly prompt rise and that the change is significantly greater than that seen in pre-exposed fibers. Also shown is that the ArP levels fall rather promptly. Such results are what one would expect if (i) ADP acts as a positive effector of phosphofructokinase, thus bringing about relief of inhibition of the enzyme by ATP (more so in pre-exposed fibers), and (ii) ADPMg acts by accelerating the forward reaction of arginine kinase.

Time-course of ATP and ArP levels in unexposed and pre-exposed fibers suspended in adenosine-ASW and injected with ADP

Summarized in Fig. 8 are the results obtained by injecting 0.1 M ADP into unexposed and pre-exposed fibers in ASW containing 1 mM adenosine. They are essentially the same as those reported in the preceding experiments. It therefore seems that regardless of whether adenosine is added or not, the effect of aldosterone is to reduce the magnitude of the change in ATP and ArP levels following a sudden rise in myoplasmic ADP concentration. Clearly, this is possible if aldosterone is, in fact, able to raise the internal level of ATP in these fibers.

Time-course of ATP and ArP levels in fibers exposed to aldosterone and cycloheximide and then injected with ADP

One way of excluding the possibility that aldosterone acts by bringing about the *de novo* synthesis of a

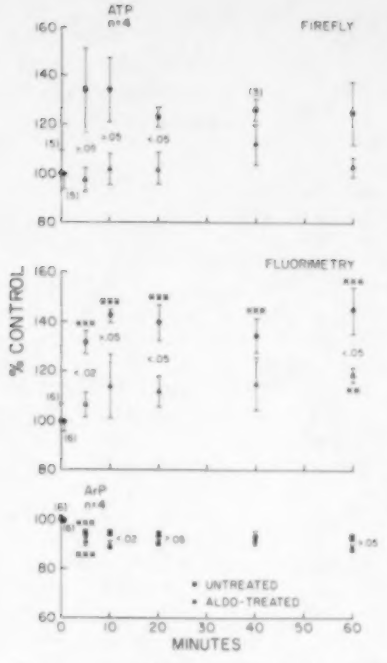

Fig. 8. Time-course of internal ATP and ArP levels in unexposed (●) and pre-exposed (▲) fibers suspended in 10^{-3} M adenosine-ASW and injected with 0.1 M ADP at $t = 0$. Upper panel: ATP values obtained with firefly. Middle panel: ATP values obtained with the fluorimetric method. Lower panel: ArP values obtained with the fluorimetric method. Unless stated otherwise in parenthesis, the number of measurements done is that given in the left upper and lower corners of the panels. Vertical bars indicate SEM. Pre-exposed fibers were isolated from the same barnacle specimen, while unexposed fibers were isolated from another specimen belonging to the same batch of barnacles then available in the aquarium. * Denotes significant difference at $P < 0.05$. ** Denotes significant difference at $P < 0.02$. *** Denotes significant difference at $P < 0.01$.

protein which suppresses the step mediating the equilibration of ADP and ATP, e.g. adenylate kinase is to use cycloheximide. Experiments therefore were carried out in which 0.1 M ADP was injected into fibers pre-exposed overnight *in vivo* to 10^{-6}M aldosterone and 10 μg/ml cycloheximide. As shown in Fig. 9, injected ADP caused the expected dual effect: it raised the internal ATP levels and it also reduced the ArP levels.

DISCUSSION

The results of this work are of interest for several reasons. First, they provide evidence that increased sensitivity to injected GTP develops fully some 8 hr after the application of aldosterone and that the effect of the steroid is neither at the transcriptional nor the translational level. The obvious implication, then, is that posttranslational control is the level at which aldosterone acts. Secondly, the basis of the enhancing effect of aldosterone does not seem to be at the level of PDE or cAMP-protein kinase, since earlier experiments involving the injection of cAMP into pre-exposed fibers (Nwoga and Bittar, 1983) and repetition of these experiments by preinjecting EDTA have failed to produce evidence of mimicry by cAMP of the kinetics seen with GTP. Thirdly, the possibility that aldosterone's site of action is calmodulin/Ca-dependent protein kinase was explored by raising K_p. This manoeuver resulted in a transitory response similar in size to that found in unexposed fibers. Fourthly, the possiblility seems real that the calmodulin form of adenylate cyclase plays a key role in the mechanism of both the increased and sustained response. This partly stems from earlier work (Bittar and Nwoga, 1981; Nwoga and Bittar, 1983) and now from the observation that imipramine is able to abolish the response to GTP. This argument, however, is valid only if imipramine acts as a specific inhibitor of calmodulin and if barnacle fibers possess calmodulin-sensitive adenylate cyclase. Thus far, brain tissue is the sole tissue reported to have this form of calmodulin, but that it will be found in due course to be ubiquitous is predicted by Brostrom and Wolff (1981). And fifthly, the experiments with cholera toxin have not yielded insight into the problem of how aldosterone acts at the level of the membrane adenylate cyclase complex. Nonetheless, the fact that the response to EDTA is considerably smaller than that caused by GTP, yet sustained, is an indication that the sensitivity of pre-exposed fibers to GTP depends not only on a fall in myoplasmic pCa resulting from CA^{2+} channel activation (following internal free Mg^{2+} removal) but also on the availability of extra GTPMg. Expressed in another way, the kinetics of the response to GTP are thought to be determined by at least three factors: a reduced pCa, newly available GTPMg and the presence of calmodulin-sensitive adenylate cyclase. Implicit, of course, is the notion that aldosterone acts by promoting the interaction of calmodulin with the catalytic subunit of adenylate cyclase.

It has long been conjectured that one of the primary functions of aldosterone is to increase cell ATP. The use of barnacle fibers as a preparation has yielded information about this possibility and has

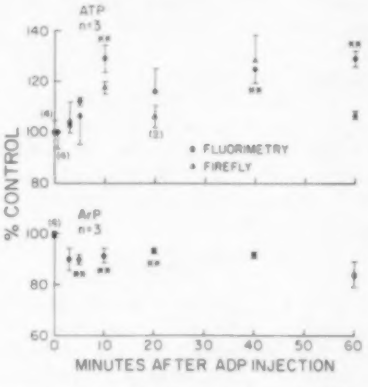

Fig. 9. Time-course of the effect on internal ATP and ArP levels of injecting 0.1 M ADP into fibers pre-exposed to 10^{-6}M aldosterone and 10μg/ml cycloheximide externally overnight. Upper panel: ATP values obtained with fluorimetric method, ●; ATP values obtained with firefly, ▲. Lower panel: ArP values obtained with fluorimetric method. Unless stated otherwise in parentheses, the number of measurements done is that given in the left upper corner of each panel. Vertical bars indicate SEM. The fibers used were isolated from the same barnacle specimen. ** Denotes significant difference at $P < 0.02$.

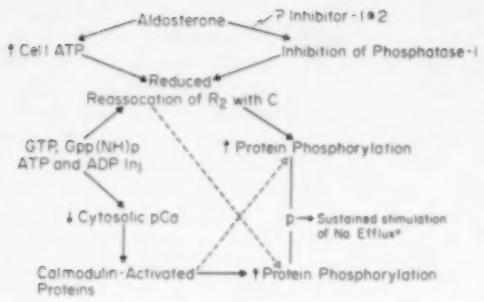

Fig. 10. Schematic representation of the way in which aldosterone and GTP [and Gpp(NH)p] are postulated to act. Note that the role of GTP as an effector of adenylate cyclase via GTPase is not shown here and that the involvement of protein inhibitor 1 and 2, as well as phosphatase 1 is purely speculative.

brought to light evidence of aldosterone raising the internal ATP level by a mechanism of activation or unmasking of proteins rather than induction. Interest in this finding is heightened especially by evidence coming from the work of Rangel-Aldao and Rosen (1977) using cAMP-protein kinase that the effect of a raised ATP level is to impede the reassociation of the regulatory subunit with the catalytic subunit, thus leading to a raised and more persistent level of catalytic activity of protein kinase. Bearing this in mind, it would be an over-simplification to think of an explanation of the kinetics of the response to GTP only in terms of pCa, GTPMg complex and calmodulin. For the present, a reasonable hypothesis is that set forth in Fig. 10. As can be seen, the scheme assumes reduced reassociation of R_2 with C as the result of a raised internal ATP level. Moreover, maneuvers which have been found to cause sustained stimulation in pre-exposed fibers, e.g. injection of guanine and adenine nucleotides, are shown in this scheme to slow the reassociation of R_2 with C. Justification for such reasoning is also to be found in the paper by Rangel-Aldao and Rosen (1977) who reported that the effect of ATP is mimicked by ADP or GTP. It should be borne in mind, however, that guanine nucleotides exert a far larger effect on the ouabain-insensitive Na efflux in pre-exposed fibers than ATP, App(NH)p or ADP (Nwoga and Bittar, 1983; E. E. Bittar and J. Nwoga, unpublished). Such a difference is attributable to the ability of GTP and Gpp(NH)p to act as effectors of adenylate cyclase activity through the GTPase system, a role not indicated in Fig. 10. This picture, where a fall in pCa, availability of extra-guanine nucleotide, together with the calmodulin form of adenylate cyclase govern the kinetics of the response to injected guainine nucleotide is too one-sided a view of the problem. The other side is to address the question whether the increased efflux (and its sustained nature) is mediated by membrane protein phosphorylation and whether the process and level of dephosphorylation is controlled by phosphatase 1, and in turn, by protein inhibitor 1 and 2. In the former case, preliminary studies have provided evidence of increased protein phosphorylation in preexposed fibers (Bárány *et al.*, 1981). In the latter case, it is now well established that skeletal muscle, e.g. rabbit muscle, possesses a Mg–ATP-dependent phosphatase (Yang *et al.*, 1980; Vandenheede *et al.*, 1980) which is inhibited by protein 1 and protein 2 (Stewart *et al.*, 1981), inhibitor 1 being only in the active form following phosphorylation by cAMP-protein kinase. Information about this in barnacle muscle and about the possibility of aldosterone packing itself in the membrane in such a way as to raise the resting level of phosphorylation as the result of suppressing phosphatase 1 or activating its inhibitors, is still completely lacking.

It may be asked, finally: does the present information about pre-exposed fibers have any bearing on the physiological problem of the permissive action of aldosterone, e.g. the ability of mineralocorticoids (and glucocorticoids) to augment the response of the kidney to vasopressin (Lang and Edelman, 1972; Stoff *et al.*, 1972; Rajerson *et al.*, 1974; Schwartz and Kokko, 1980)? In a word, "yes", for it is tempting to argue that aldosterone acts in ductal "principal" cells in two ways: one is by promoting the involvement of the calmodulin form of adenylate cyclase, and the other by increasing the internal ATP, thereby delaying the reassociation of cAMP-protein kinase holoenzyme.

Acknowledgement—This work was supported in part by grants from the Graduate School Research Committee and the National Science Foundation.

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RESPONSES OF PHOTOSYNTHESIS, RESPIRATION, GROWTH AND CELLULAR CONSTITUENTS TO HYPO-OSMOTIC SHOCK IN THE RED ALGA *GRACILARIA TIKVAHIAE*

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Abstract—1. A short-term (hours–2 days) transient decrease in photosynthesis and increase in dark respiration accompanied hypo-osmotic shock from 26 to 16‰ salinity in *G. tikvahiae*.

2. Growth rate of *G. tikvahiae* equilibrated to 16‰ was equal that at 26‰, supporting classification of this alga as a euryhaline species.

3. Levels of free amino acids were the same in *G. tikvahiae* grown at 26 and 16‰; levels of ash were markedly different and suggests osmotic adjustment is achieved primarily through inorganic solutes.

4. Levels of six ions (K^+, Na^+, Ca^{2+}, Mg^{2+}, Cl^-, SO_4^{2-}) collectively constitute *ca* 97% of the ash of *G. tikvahiae* at both salinities, only levels of Cl^- and Ca^{2+} were proportional to salinity and, relative to dissolved components of seawater, only K^+ is enriched in the ash.

5. An apparent excess of inorganic anions in *G. tikvahiae* at 16‰ may be a biochemical response to displace the higher influx of protons from a less saline, more acidic medium; such a response would explain, in part, the ecological success of *G. tikvahiae* in shallow estuaries where wide salinity fluctuations are common.

INTRODUCTION

The effects of salinity on growth and physiological processes of marine algae are of considerable complexity. Most experimental investigations use seawater diluted with distilled or tap water to achieve various water salinities. As Ogata and Matsui (1965) pointed out: "It may generally be said that the changes in salinity, osmotic pressure, pH and also carbon dioxide supply, particularly in natural seawater, are rather inseparably associated." Consequently, few studies have demonstrated unequivocal, general relationships between salinity and various physiological responses in marine algae. The few studies on relations between respiratory rates and salinity show little agreement (Gessner and Schramm, 1971). Gessner and Schramm (1971) concluded that effects of salinity on respiratory rates of marine algae are primarily affected by oxygen supply, which covaries with salinity. Only in extreme low or high salinities may osmotic processes influence algal respiration (Ogata, 1963). Similarly, relations between photosynthesis, growth and salinity are contradictory and have not led to generally accepted conclusions (Gessner and Schramm, 1971).

Although osmoregulation and osmoaccumulation in algae rely primarily on ion pumps (Gutknecht and Dainty, 1968; MacRobbie, 1974) various algae respond to salinity changes by altering concentrations of osmotically active organic substances—a phenomenon that occurs in phylogenetically diverse organisms, including bacteria, plants and animals (see Yancey *et al.*, 1982, for review). Following hyperosmotic shock, *Chlorella fusca* accumulated oligosaccharides (Dedio, 1968) and *Monochrysis lutheri* increased its concentration of cyclohexanetetrol (Craigie, 1969). Sorbitol and proline are osmotically active in *Stichococcus bacillaris* (Brown and Hellebust, 1980) and free amino acids decreased when the red alga *Porphyridium purpureum* was exposed to hypo-osmotic conditions (Gilles and Pequeux, 1977).

The present study investigated the effects of hypo-osmotic shock on growth, photosynthesis and dark respiration of the red alga *Gracilaria tikvahiae* (McLachlin, 1979). This hypo-osmotic shock simulates rapid decreases in salinity (e.g. within hours) which occur frequently in shallow estuaries and coastal marine ecosystems, particularly in subtropical and tropical latitudes. Changes in cellular constituents (% dry matter, % ash, free amino acids, major inorganic ions) during the hypo-osmotic shock were also monitored to determine the relative importance of free amino acids and inorganic ions as osmotically active solutes in *G. tikvahiae*.

MATERIALS AND METHODS

Stock cultures of *G. tikvahiae* (clone Orca) were used for this experiment between 1 and 13 May, 1981, in outdoor continuous cultures (see Lapointe and Tenore, 1981, for description of culture chambers). *G. tikvahiae* from the stock culture (26‰) was inoculated into four experimental chambers; two replicate chambers were maintained at ambient salinity (26 ± 2‰) and two at a reduced level (16 ± 2‰). Each culture chamber received 5-μ filtered, temperature-regulated (21°C ± 2‰) seawater metered by PVC chemcock

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values at a flow rate of 500 ml·min^{-1} (turnover rate of 15 chamber volumes·day^{-1}). The reduced salinity treatments received freshwater (wellwater) in addition to seawater to reduce salinity. This experimental design minimizes the confounding effects of reduced carbon supply in reduced salinity treatments (Ogata and Matsui, 1965) by providing high turnover rates of seawater. All chambers received nutrient-enrichment from medium ($NaNO_3$, NaH_2PO_4) metered to the chambers by single-channel peristaltic pumps. Salinity was measured daily with an optical refractometer and varied by no more than ±5‰.

Changes in the wet weight (damp-dried) of *Gracilaria* were used to calculate specific growth rates (doublings·day^{-1}) as:

$$\mu = \frac{\log_{10} \frac{B}{B_0} 3.32}{\Delta t}$$

where B_0 is the initial biomass and B is the final biomass with time (t). Algal density was maintained at 100 g·chamber^{-1} (wet weight) by harvesting at each growth period. At the end of the experiment (12th day), algal samples were dried at 85°C for 48 hr to determine % dry weight and combusted at 475°C for 12 hr to determine % ash. Free amino acids were estimated using the fluorescamine protein–amino acid technique (Udenfriend *et al.*, 1972), on ground lyophilized samples of *G. tikvahiae*. This method was modified with a protein precipitation step (cold TCA) to estimate free amino acids as protein equivalents.

Ground lyophilized samples were also used for determinations of both total and water-soluble major ions (Na^+, K^+, Mg^{2+}, Ca^{2+}, SO_4^{2-} and halide (principally Cl^- but including minor Br^- and I^-). For total assay of metals and sulfate, duplicate samples (0.100 ± 0.020 g) were ashed in sub-boiling reagent-grade nitric acid in tightly capped teflon vessels; the dry residual ash was redissolved in 1N nitric acid. Total halide and water-soluble metals and sulfate were extracted from other duplicate 0.1 g samples (10 ml distilled water, initial pH = 6.97, 25 ± 2°C, extraction time 3 hr); residual solids were removed by pressure filtration through acid-washed glass-fiber filter (*ca* 1-μm particle retention). Solutions from both treatments were diluted with distilled water as required and chemically assayed as described below.

Sodium and potassium were determined by atomic emission spectroscopy; magnesium and calcium were determined by atomic absorption spectroscopy (Windom and Cutshall, 1976). Sulfate was measured by barium sulfate turbidimetry (2.5 cm photometer cell, 420 nm radiation) (Thomas and Cotton, 1954). Halides were determined by argentiometric titration using potassium chromate for endpoint detection. As a check on analytical accuracy and precision, samples of NBS Reference Material (orchard leaves) and procedural blanks (10 ml of nitric acid or distilled water) were taken through the preparation procedure. elemental determinations on the orchard leaves were within the ranges of certified values; blank values were negligible at the level of the analysis.

Rates of photosynthesis and dark respiration were monitored for 96 hr after exposure to the salinity treatments by using light and dark bottle O_2 techniques. Whole plants (2–5 g wet wt) were incubated in 500 ml B.O.D. bottles *in situ* in the experimental chambers using seawater from the chamber under study. All measurements were taken over short time periods (e.g. 1–2 hr) to eliminate bottle effects associated with long term incubations. For photosynthetic measurements, the seawater was initially bubbled with N_2 to reduce O_2 levels to *ca* 1 ppm to avoid bubble formation that occurs at O_2 levels near saturation. All B.O.D. bottles were mixed using stirbars and air-driven submersible magnetic stirrers. Three replicates for both light and dark bottles were used as well as light and dark bottle blanks. O_2 levels were measured with a Beckman Field Lab 100 800 oxygen analyzer equipped with a polarographic electrode. The O_2 analyzer was calibrated using an oxygen-free solution and air-saturated seawater of known temperature and salinity. Rates of photosynthesis and dark respiration, measured as described above, were found to be linear over the time course of the incubation periods used.

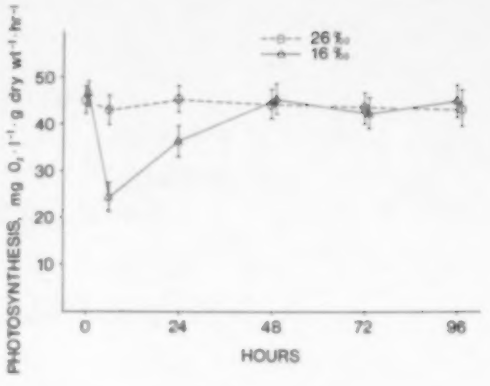

Fig. 1. Time course of photosynthesis in *Gracilaria tikvahiae* exposed (at 0 hr) to ambient salinity (26‰) and a hypo-osmotic shock (26→16‰). Plants were previously growing at 26‰ salinity. Values represent means ± 1 SD ($N = 3$).

RESULTS

Photosynthetic and respiratory rates of *G. tikvahiae* exposed to hypo-osmotic shock (16‰) were significantly ($P < 0.05$) different from those of plants maintained at ambient salinity (26‰) during the first 24 hr of exposure (Figs 1 and 2). Photosynthetic rates decreased by *ca* 50% after 4 hr exposure to the lowered salinity. Respiratory rates of *G. tikvahiae* increased by *ca* 37%, after 4 hr exposure to lowered salinity (Fig. 2). By 48 hr after exposure photosynthetic and respiratory rates of *G. tikvahiae* at the different salinities were similar (Figs 1 and 2).

No significant difference in growth rates of *G. tikvahiae* at the different salinities were detected over 12 days of monitoring growth (Fig. 3).

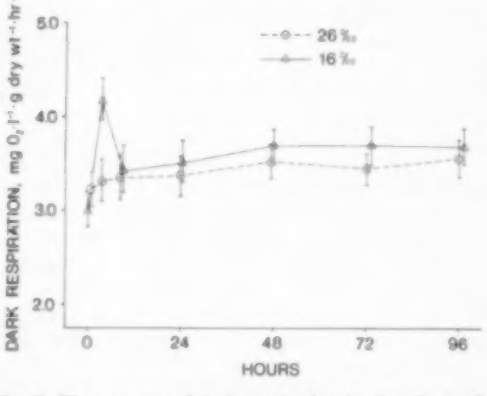

Fig. 2. Time course of dark respiration in *Gracilaria tikvahiae* exposed (at 0 hr) to ambient salinity (26‰) and a hypo-osmotic shock (26→16‰). Plants were previously growing at 26‰ salinity. Values represent means ± 1 SD ($N = 3$).

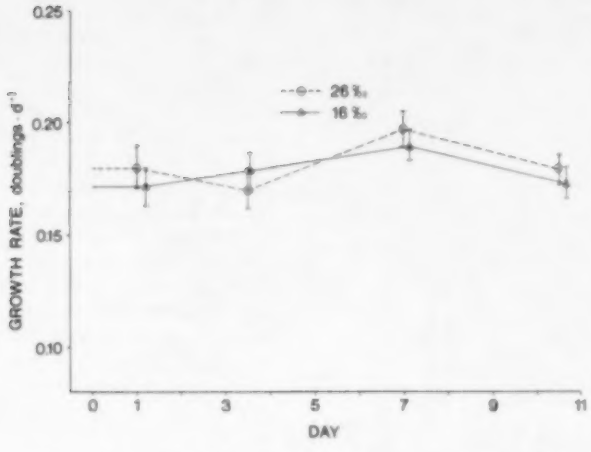

Fig. 3. Time course of growth rate of *Gracilaria tikvahiae* exposed to ambient salinity (26‰) and a hypo-osmotic shock (16‰) on day 0. Plants were previously growing at 26‰ salinity. Values represent means ± 1 SD ($N = 2$).

The % dry weight and ash level of *G. tikvahiae* exposed to the hypo-osmotic shock was different from that of *G. tikvahiae* at ambient salinity at the end of the experiment. *G. tikvahiae* exposed to 16‰ salinity had significantly ($P < 0.05$) greater percent dry weight and significantly ($P < 0.05$) less ash than *G. tikvahiae* at 26‰ salinity (Table 1). The free amino acid levels of *G. tikvahiae* from the two treatments at the end of the experiment were not significantly different ($P > 0.05$; Table 1).

With the exception of sulfate, levels of the measured elements were higher in the *G. tikvahiae* grown at ambient salinity (Table 2). Chloride (more correctly, halide) and calcium levels were proportional to salinity; however, no proportional relationship between elemental level in *G. tikvahiae* and salinity were observed for the other elements. Element/chloride ratios (Table 2) indicate that, relative to the dissolved components of seawater, only potassium is enriched in the ash of *G. tikvahiae*. K^+/Cl^- and SO_4^{2-}/Cl^- ratios were significantly lower and the Na^+/Cl^- ratio significantly higher in *G. tikvahiae* grown at ambient salinity (Table 2).

Essentially all of the sodium, potassium, calcium and (presumably) halides may be removed by simple leaching in distilled water without destruction of the organic matrix (Table 2). Approximately 20% of the magnesium and 25% of the sulfate were not removed by treatment with distilled water.

The ion electrical balance, based on total levels of the seven metal species (Table 3), demonstrates electrical neutrality in *G. tikvahiae* at 26‰ and an apparent, significant excess of inorganic anions in the *G. tikvahiae* at 16‰. Charge balances for water extractable species suggests electrical neutrality (Table 3).

DISCUSSION

The overall lack of long-term (days–weeks) effects of reduced salinity on growth and photosynthesis supports the classification of this alga as a euryhaline species. Conover (1964), in a study of salinity requirements of 74 species of marine vascular plants and algae in Texas lagoons, found only 9 euryhaline species including *Gracilaria foliifera v. angustissima* (= *G. tikvahiae*, see McLachlin, 1979), which was collected in salinities ranging from 7 to 43‰. The lack of salinity effects on growth rate, at least in the range of the present study, is also supported by outdoor continuous-culture studies. Maximum growth rates of *G. tikvahiae* grown at 25°C under maximum levels of natural light intensity with nutrient enrichment at 19‰ were 0.37 ± 0.07 doublings·day^{-1} (Lapointe, 1981). More recent studies, using the same clone of *G. tikvahiae* and grown under identical light, temperature and nurient conditions as in the previous study except for elevated salinity which was 29‰, also reported maximum growth rates of 0.37 ± 0.02 (Lapointe *et al.*, 1983). Inasmuch as vegetative growth of mature plants of *G. tikvahiae* is important in the life history of this alga (Humm, 1944), the ability of *G. tikvahiae* to maintain maximum growth rates over a broad range of salinities must be viewed

Table 1. Levels of dry weight (in terms of wet weight), ash (in terms of dry weight), and free amino acids (in terms of % protein equivalents and corrected for water level of the sample) of *Gracilaria tikvahiae* cultured at different salinities

Salinity (‰)	% Dry weight	% Ash	Free amino acids
16	11.0 ± 0.2	34.9 ± 1.0	5.1 ± 0.3 (= 0.82% N)
26	10.1 ± 0.1	45.9 ± 1.0	5.0 ± 0.2 (= 0.80% N)

Values represent means ± 1 SD ($N = 5$).

Table 2. Inorganic composition of *Gracilaria tikvahiae* grown at two salinities (16 and 26‰)

	Composition of *Gracilaria* mmol·g dry wt^{-1}				Element:halogen ratio *Gracilaria*		Percent water •extractable	
	$S = 16$‰	$S = 26$‰	Ratio X_{26}/X_{16}	Seawater•	$S = 16$‰	$S = 26$‰	$S = 16$‰	$S = 26$‰
Na	0.75 ± 0.01	1.73 ± 0.05	2.30 ± 0.08	0.85	0.26 ± 0.01	0.38 ± 0.01	95 ± 7	98 ± 4
K	3.04 ± 0.05	3.84 ± 0.03	1.26 ± 0.02	0.019	1.07 ± 0.01	0.84 ± 0.01	95 ± 6	97 ± 1
Mg	0.13 ± 0.01	0.24 ± 0.01	0.85 ± 0.01	0.097	0.047 ± 0.004	0.052 ± 0.001	77 ± 7	84 ± 2
Ca	0.063 ± 0.001	0.099 ± 0.002	1.57 ± 0.03	0.019	0.022 ± 0.001	0.19 ± 0.001	92 ± 8	95 ± 4
Cl	2.85 ± 0.08	4.57 ± 0.06	1.60 ± 0.05	1	1	1	100	100
SO_4	0.97 ± 0.04	0.90 ± 0.03	0.93 ± 0.05	0.052	0.34 ± 0.02	0.20 ± 0.01	73 ± 5	74 ± 12

Composition is variously expressed as total concentrations, relative concentrations in plants at different salinities (X_{26}/X_{16}), concentrations relative to total halides (as Cl^-), and as percent of total concentration which may be extracted by treatment with distilled water. Uncertainties are expressed as ± 1 SD. (Ratios calculated with uncertainties propagated according to Bevington, 1969).

•After Brewer (1975).

as an important ecological adaptation of this species.

Our observed short-term increase in dark respiration following osmotic shock may reflect an energy requirement for osmoregulation in *G. tikvahiae*. There is little quantitative information for marine algae regarding ionic concentrations in the cytoplasm and vacuoles, the magnitudes of the ion fluxes and the electrical potential differences across the membranes. Consequently, it is difficult to estimate the energy requirements of the pumps. As suggested by Jeffries (1982), a euryhaline cell that experiences changing salinity regimes will require additional energy to cope with transient physiological processes involved in osmoregulation. Kesseler (1965) also reported increased respiration of *Chaetomorpha linum* upon transfer from 50 to 20‰ salinity.

The short-term, transient decrease in photosynthesis of *G. tikvahiae* following hypo-osmotic shock may be related to exosmosis of ions required as cofactors in photosynthesis. Gessner and Hammer (1968) demonstrated that ion loss, in particular Cl^-, occurs rapidly (minutes) when marine algae are exposed to hypo-osmotic conditions. Our finding that chloride content is proportional to salinity supports the hypothesis that chloride is transported passively across the plasmalemma (MacRobbie, 1974). Thus, rapid decreases in salinity, which occur frequently in estuaries and coastal marine waters where *G. tikvahiae* is abundant, may induce transient, decreased rates of photosynthesis in this alga and perhaps other marine algae as well.

Because photosynthetic rates are often used as a physiological index for performance of marine algae exposed to different salinities, such investigations obviously need to allow adequate acclimation before assessment of photosynthetic performance. A 2–3 day acclimation period was adequate for *G. tikvahiae* within the salinity range of the present study and this compares favorably with the 4-day acclimation period suggested by Yarish *et al.* (1979) based on their studies with three estuarine red algae.

Calcium levels were also proportional to salinity in *G. tikvahiae*, a finding that supports early investigations with *Chlorella pyrenoidosa* where calcium was accumulated in proportion to the calcium concentration of the external medium (Knauss and Porter, 1954). If distilled water had been used to dilute the seawater of 26 to 6‰, the Ca_{26}/Ca_{16} ratio would probably have been closer to 1.62 (= 26 ÷ 16); because calcium is the major cation of the well water used for dilution, the slightly lower Ca_{26}/Ca_{16} of 1.57 was found.

Regulation of free amino acid levels is an important mechanism in the control of intracellular osmolarity in the animal kingdom but a parallel importance in marine algae has not been generally demonstrated. Although Gilles and Pequeux (1977) found higher levels of free amino acids in *Porphyridium purpureum* cultured in seawater compared to freshwater, levels of major inorganic ions (Na^+, K^+, Cl^-) were also much higher in their seawater cultured *P. purpureum*. In studies of osmotic adaptation of the euryhaline *Ulva lactuca* to fluctuating salinity regimes, no significant changes in free amino acids were found while potassium, sodium, chloride and sulfate varied according to salinity and the

Table 3. Inorganic ion elecrical balance in *Gracilaria tikvahiae* grown at two salinities

	Ion electrical balance in *Gracilaria* (meq·g dry wt^{-1})	
	$S = 16‰$	$S = 26‰$
Total assay		
Cations	$+4.18 \pm 0.08$	$+6.25 \pm 0.12$
Anions	-4.79 ± 0.16	-6.37 ± 0.12
Total	-0.61 ± 0.24	-0.12 ± 0.24
Water-extractable		
Cations	$+3.92 \pm 0.34$	$+9.01 \pm 0.22$
Anions	-4.27 ± 0.24	-5.91 ± 0.32
Total	-0.35 ± 0.58	$+0.10 \pm 0.54$

Balances are given for total ion assay and for water-extractable ions.

experimental treatment (Dickson *et al.*, 1982). Our results with euryhaline *G. tikvahiae* support the findings of Dickson *et al.* (1982) that free amino acids play an insignificant, or at least, minor role compared to inorganic ions in osmotic balance in euryhaline algae. By converting levels in mmol·g dry wt^{-1} to g·g dry wt^{-1} one can demonstrate that the seven ions we assayed collectively constitute $33.6 \pm 0.9\%$ and $44.7 \pm 0.7\%$ of the mass of the 16 and 26‰ *G. tikvahiae*, respectively. These values for ash level compare favorably with those determined by muffle furnace combustion (34.9 and 45.9%, respectively, Table 1) and indicate that the four metals, sulfate and halogens account for essentially all of the ash of *G. tikvahiae*. Potassium, sulfate and halogens account for 90–95% of the ash. Thus, the "sodium–potassium" pump, as originally suggested by Meyer (1891), appears to be the major process involved in osmoregulation in *G. tikvahiae*. Despite antithetical changes in Na/Cl and K/Cl ratios, (Na + K)/Cl is nearly the same at both salinities, suggesting that to some extent sodium and potassium may be metabolically interchangeable in *G. tikvahiae* (Allen, 1952).

One final point revealed by this study is an *apparent* excess of inorganic anions in *G. tikvahiae* grown at 16‰ compared to electrical neutrality at 26‰. A charge imbalance has also been reported for *Ulva lactuca*, but in that alga an excess of inorganic cations occured when equilibrated to full strength seawater (33.5‰; Dickson *et al.*, 1982). Comparison with charge balances for water-extractable species—which are electrically balanced—suggests that at lower salinities *G. tikvahiae* may contain higher amounts of sulfated structural polysaccharides that might be electrically balanced with protons. The occurrence of appreciable amounts of vacuolated free sulfuric acid in some algae (Schiff, 1982) may indicate that production of polysaccharide esters in *G. tikvahiae* at lower salinity are a biochemical response of this alga to displace the higher influx of protons from a less saline, more acidic medium. Such a biochemical strategy would explain to some extent the ecological success of *G. tikvahiae* in shallow estuaries where wide salinity fluctuations are common.

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ORGAN SCALING IN MAMMALS: THE KIDNEYS

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Abstract—1. Values of kidney weight in adult male and female mammals, both terrestrial and aquatic, as well as values for renal blood flow and glomerular number and diameter, were submitted to linear (log–log) regression analysis.

2. The slope of the regression line for kidney weight in 63 species of adult terrestrial mammals was 0.85 ± 0.01. No statistically significant difference was found between the slopes of the regression lines for male and female terrestrial mammals.

3. The slope of regression line for kidney weight in eight species of adult aquatic mammals was 0.92 ± 0.01. Again, no statistically significant difference was found between the slopes for males and females. However, the slope (0.92) of the regression line for aquatic mammals was significantly different from the slope (0.85) for terrestrial mammals ($P \ll 0.001$).

4. The slope of the regression of renal blood flow on body weight was 0.82 ± 0.02. This value is consistent with the hypothesis that renal blood flow represents a constant fraction of cardiac output (over about 3.4 orders of magnitude in body weight).

5. The slopes of the regression lines for glomerular number (per kidney) and mean glomerular diameter were 0.59 ± 0.02 and 0.11 ± 0.01, respectively.

6. A schematic model representing the scaling of energy-partitioning in mammals is introduced.

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INTRODUCTION

Living organisms require a continual flux of energy in order to sustain their vital activities. Mammals and birds contrive to maintain their resting metabolism some 29 times that of poikilotherms and 233 times that of (aggregated) single celled organisms of equal weight (Hemmingsen, 1960, p. 18). Insight into the mechanisms by which mammals and birds achieve such high levels of resting energy flux is fundamental to an understanding of their "design".

Whole body relationships between oxygen consumption—as a measure of energy metabolism—and body weight in adult mammals are well known. Studies by Bartels (1982) and Kleiber (1947), among others, have shown that energy metabolism is a power function of body weight. But the apparent simplicity of the whole body relationship disguises an underlying complexity. For example, energy metabolism is partitioned quite unequally among the different organs and tissues (e.g. Brozek and Grande, 1955, p. 27).

An understanding of organ-specific energy metabolism is an essential prerequisite to an understanding of whole body energy metabolism. Attempts have been made to understand organ and tissue energy metabolism through *in vitro* measurements, but the results of these have proved difficult to interpret, due to differences in culture media, variations in temperature, use of weight-specific units, etc. (see Davies, 1961). In the long run, *in vivo* measurements of organ blood flow and oxygen consumption hold the greatest promise of providing the requisite information.

In prior studies I have reviewed organ and tissue scaling data in mammals for liver, blood and heart (Prothero, 1979, 1980, 1982). In this study a variety of scaling data on the kidney was analyzed (see also Edwards, 1975). First, a summary of the data base is presented, followed by the regression coefficients which we have derived. Finally, some implications of the results are discussed, along with a few preliminary remarks on how the regression parameters may be integrated into a coherent model of energy-partitioning in mammals.

DATA BASE

Except where noted, all the references cited below provide measured values of a renal parameter (kidney weight, renal blood flow, number of glomeruli per kidney, mean glomerular diameter) and body weight. In judging maturity on the basis of body weight, I have followed the adult weight ranges given by Walker *et al.* (1975), Eisenberg (1981) and Whitaker (1980).

In those instances where the species name was not given but can be inferred from a common name, the species name is followed by a question mark. Where the species name cannot be reliably inferred from a common name, I have used the designation SU (species unidentified).

Kidney weight

The bulk of the data on kidney size in adult mammals are taken from Crile and Quiring (1940). I have omitted data pertaining to animals with pathology (tuberculosis, goitre, pneumonia, etc.), as well as geldings, and pregnant females. Likewise, I have omitted organs for which only preserved weights are given. The data for shrews (*Suncus etruscus* and *Crocidura russula*) are taken from Bartels *et al.* (1979). The data for kidney weight in *Cetacea*, *Sirenia* and *Pinnipedia* come from the same sources as cited in a previous paper (Prothero, 1982).

Renal blood flow

The data on renal blood flow were obtained from the following sources.

Artiodactyla. Renal blood data in the sheep (*Ovis aries* ?) are provided by Rosenfeld (1977).

Carnivora. For data on dogs (*Canis familiaris* ?) see Sapirstein (1958) and Levy and Blalock (1937).

Table 1. Kidney weight as a function of body weight in adult mammals*

Habitat	Sex	Orders	Species	Animals	Points	*pWR*	*a*	log ± SE	*b* ± SE	*r*	% Dev
Terrestrial	M	8	39	107	56	5.6	0.019	−1.73 ± 0.02	0.87 ± 0.02	0.992	27.5
	F	4	14	693	31	4.8	0.021	−1.67 ± 0.02	0.84 ± 0.02	0.994	22.1
	B	11	63	1105	117	6.4	0.020	−1.69 ± 0.01	0.85 ± 0.01	0.993	25.3
Aquatic	M	1	7	25	22	3.6	0.017	−1.76 ± 0.01	0.92 ± 0.01	0.998	10.8
	F	2	5	29	28	3.7	0.015	−1.82 ± 0.01	0.92 ± 0.01	0.998	13.4
	B	2	8	52	48	3.7	0.0161	−1.78 ± 0.01	0.92 ± 0.01	0.998	12.6

*The model is $y = ax^b$ where x is the variate (body weight), y is the covariant (e.g. kidney wt), a (intercept) and b (slope) are the regression coefficients, r is the correlation coefficient and pWR is the logarithm of the weight ratio. Percent deviation is the mean deviation from the least squares regression line. See text.

Lagomorpha. For data on the rabbit (SU) see Jones and Parker (1980) (absolute blood flows by personal communication) and Neutze *et al.* (1968).

Pinnipedia. Renal blood flows in the Weddell seal (*Leptonychotes weddelli*) are given by Zapol *et al.* (1979).

Primates. Data for the baboon (*Papio anubis*) are presented by Vatner (1974). For data on human renal blood flow see Bolomey *et al.* (1949), Brun *et al.* (1955), Goldring *et al.* (1940) and Ladefoged and Pedersen (1967).

Rodentia. For data on the rat (*Rattus norvegicus* ?) refer to Goldman (1966), Ishise *et al.* (1980), Jansky and Hart (1968), Mandel and Sapirstein (1962), McDevitt and Nies (1976), Sapirstein *et al.* (1960), Takacs (1965), Takacs *et al.* (1961), Takacs and Vajda (1963) and Tsuchiya *et al.* (1978). Gjedde and Gjedde (1980) provide renal blood flow data for the mouse (*Mus musculus*).

Kidney structure

Data on the number of glomeruli per kidney, the mean diameter of the glomeruli or both are available as follows:

Carnivora. Finco and Duncan (1972), Kunkel (1930) and Vimptrup (1928) provide data for the dog (*Canis familiaris* ?). Vimptrup (1928) also gives data for the cat (*Felis cattus* ?).

Lagomorpha. For data on the rabbit (SU) see Knepper *et al.* (1977) and Moritz and Hayman (1934).

Marsupialia. Bentley and Shield (1962) supply data for *Setonix brachypus*.

Primates. For data on monkey (SU) see Kunkel (1930). For man consult Moritz and Hayman (1934) and Vimptrup (1928).

Proboscidea. For the elephant (SU) see Rytand (1937).

Rodentia. Data for the rat (*Rattus norvegicus* ?) are furnished by Arataki (1926), Kaufman *et al.* (1975), Kaufman *et al.* (1976), Knepper *et al.* (1977), Moore (1929), Rytand (1937) and Vimptrup (1928). For the mouse (*Mus musculus* ?) see Rytand (1937). Data for the guinea pig (*Cavia porcellus* ?) are reported by Kunkel (1930). For the kangaroo rat (SU) see Rytand (1937). Kunkel (1930) also studied the ground hog (SU).

REGRESSION ANALYSIS

The data were expressed in logarithmic form and linear least squares regression analyses performed (Edwards, 1975). For each regression line a mean percent deviation (PD) between the data points and the regression line was calculated from the equations:

$$y_i' = x_i^b$$

$$PD = \frac{100}{n} \sum_{i=1}^{n} |y_i - y_i'|$$

where x_i is the body weight (g) of the ith animal, y_i is the covariant (e.g. kidney weight), y_i' is the value of the covariant predicted by the regression line. n is the number of data points.

The standard error in the regression coefficients depends chiefly upon the weight range spanned by the data. A helpful measure of the weight range is given by:

$$pWR = \log(\text{max wt/min wt})$$

i.e. the logarithm of the ratio of the largest to the small body weight found in the given data set. From shrew to blue whale, the value of pWR is about 8. For terrestrial mammals (shrew to African elephant), pWR is at least 6.5, whereas for aquatic mammals (dolphin to blue whale), pWR is only about 4.8.

RESULTS

Regression parameters for kidney weight as a function of sex and habitat in adult mammals are given in Table 1.* The regression lines for aquatic and terrestrial mammals and the data for terrestrial mammals are shown in Fig. 1. Because of the overlap in the data, the regression line and data for the aquatic mammals are presented separately in Fig. 2.

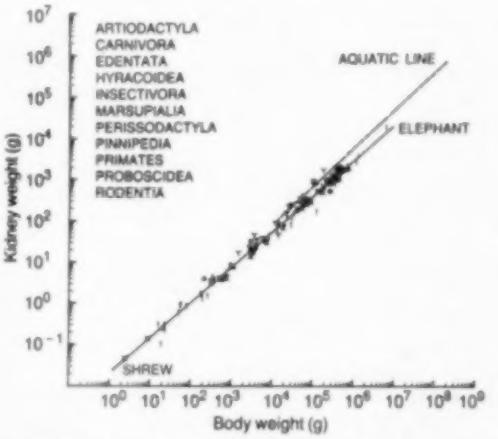

Fig. 1. Kidney weight in adult mammals. The solid line, with slope 0.85, applies to terrestrial mammals. The dashed line, with slope 0.92, applies to aquatic mammals. The 11 orders, embracing 63 species represented in the data, are also indicated.

*The data from which the regression coefficients given in Table 1 were derived, along with the bibliographic sources, have been deposited as Document No. 04106 with the National Auxiliary Publications Service of the American Society for Information Science, c/o Microfiche Publications, Division of Microfiche Systems Corporation, P.O. Box 3513, Grand Central Station, New York, N.Y. 10017, U.S.A.

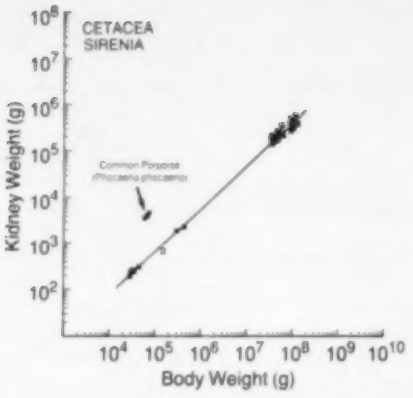

Fig. 2. Kidney weight in adult mammals. The slope of the regression line is 0.92. The data for the common porpoise were not included in the calculation of the regression. Two orders and eight species are represented in the data line. See also Fig. 1.

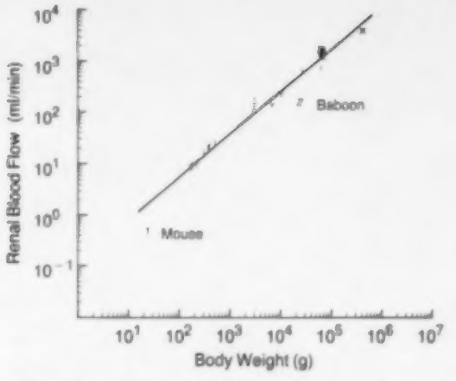

Fig. 3. Renal blood flow (RBF) in adult mammals. The slope of the regression line is 0.82. The values of RBF for the mouse and baboon were not included in the regression calculation.

The regression coefficients for renal blood flow and two aspects of kidney structure are presented in Table 2. A plot of renal blood flow versus body weight is given in Fig. 3.

DISCUSSION OF REGRESSION ANALYSES

Kidney weight in adult mammals

Regression parameters for kidney weight on body weight in adult terrestrial mammals of both sexes are given in Table 1 (rows 1 and 2). There is no significant difference in the slopes of the regression lines as between male and female mammals ($d.f. = 83, t = 1.07, P < 0.2$). The regression parameters of adult aquatic mammals (*Cetacea* and *Sirenia*) for males and females are given in rows 4 and 5 of Table 1. Again there is no significant difference in the slope of the regression lines for males and females ($d.f. = 56, t = 0.35, P < 0.5$).

However, there is a highly significant difference between the slope (0.85) for the terrestrial mammals and the slope (0.92) for aquatic mammals ($d.f. = 173$, $t = 3.7$, $P \ll 0.001$) (see Table 1, rows 3 and 6). In calculating the regression lines for aquatic mammals, I have omitted the data for the common porpoise (*Phocaena phocaena*). The kidney weights for these mammals are 89% above the regression line for the other aquatic mammals (see Fig 2).

The values for the weight-coefficient (0.020) and the slope (0.85) obtained here for the regression of kidney weight on body weight in adult terrestrial mammals are essentially identical to the values (0.021 and 0.85, in our units, respectively) obtained by Brody (1945), who used a much smaller data base. The regression parameters for kidney weight on body weight in adult aquatic mammals (see Table 1, rows 4, 5 and 6) are reported here for the first time (apparently).

It is unclear why kidney weight in the common porpoise should exceed that for dolphins, which have a similar body weight and diet, by as much as 89% (see Fig. 2). Further determinations of kidney weight in porpoises would be of interest.

Renal blood flow (RBF)

The slope of the regression line of RBF on body weight ($pWR = 3.4$) is 0.82 ± 0.02 (see Table 2, row 1 and Fig. 3). The mean deviation about the regression line is 18.5%. Holt *et al.* (1968) have shown that cardiac output in terrestrial mammals varies as the 0.79 power of body weight ($pWR = 3.3$). The slope obtained here for RBF on body weight is not significantly different from 0.79 ($d.f. = 19$, $t = 1.7$, $P < 0.1$). This result is consistent with the hypothesis that RBF, over the weight range given here ($pWR = 3.4$), is a constant fraction of cardiac output. Edwards (1975) reached a similar conclusion (for $pWR = 2.2$).

Table 2. Various renal scaling parameters in adult animals

Parameter	Orders	Species	Animals	Points	pWR	a	log ± SE	b ± SE	r	% Dev
Renal blood flow (ml/min)	6	6	205	21	3.4	0.12	−0.92 ± 0.03	0.824 ± 0.02	0.994	18.5
Number of glomeruli (per kidney)	7	8	84	52	5.4	1.5×10^4	4.18 ± 0.02	0.59 ± 0.02	0.983	22.9
Mean glomerular diameter (micrometers) μm	5	5	39	39	5.4	61.0	1.78 ± 0.01	0.11 ± 0.01	0.975	7.4

See footnote to Table 1.

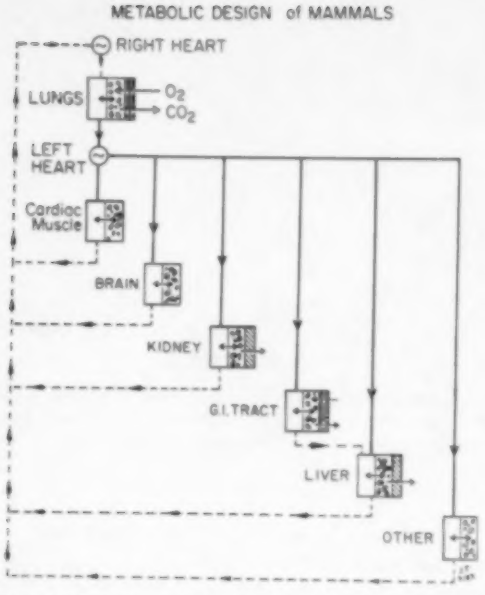

Fig. 4. A schematic model of energy partitioning in mammals. Organs and tissues are represented by compartments. Fluxes occur between the blood and the tissues as well as between the tissues and the external environment. The left and right hearts are represented by idealized pumps, which consume no energy. Cardiac muscle and the other tissues are sinks for oxygen and sources for carbon dioxide. Steady-state energy partitioning can be described, in the first instance, in terms of the oxygen unloaded per unit time in each compartment. As a rough approximation, the exchanges with the environment, apart from respiration, can be neglected. In general, the fluxes for each compartment vary systematically with organ size.

Glomerular number and diameter

The slopes of the regression lines relating the number of glomeruli (per kidney) and mean glomerular diameter to body weight are 0.59 ± 0.02 and 0.11 ± 0.01, respectively.

Smith (1951, p. 570), following Rytand (1937), concluded that the total glomerular volume varies as nearly the first (i.e. 1.024) power of kidney weight. In checking this calculation, I obtained essentially the same slope, but the SE proves to be rather large (± 0.04). The mean deviation from the regression line is 31.5%.

GENERAL DISCUSSION

An obvious starting point in constructing a scalable model of energy-partitioning in mammals is the familiar lumped-parameter model of the circulatory system (e.g. Guyton *et al.*, 1972). But such a model is seemingly too simple, since it neglects all exchanges between the organism and the environment. A more realistic model results from making allowance for specific exchanges, as appropriate, between each organ or tissue and the external environment (see Fig. 4) (thermal exchanges, however, are not represented in the present version of the model).

In this model there are "control" surfaces associated with a number of the organs (e.g. gastro–intestinal tract, kidney, lungs) which regulate the exchange of materials between the internal and external environments.

In principle, correct (proper) physical equations can be written down describing the behavior of a model such as that illustrated in Fig. 4. The steady-state fluxes between the blood and the tissues and across the "control" surfaces will be a function of surface areas, permeability coefficients, concentration gradients, etc. (e.g. see Bischoff, 1967; Holmgren, 1966).

Unfortunately, the parameters which appear in the physical equations, such as surface area, are either currently unknown or known in only a few restricted instances. Thus there is, at present, very little prospect of constructing an empirically-based scalable model of mammalian energy-partitioning by a direct attack on the problem.

One strategy to surmount this difficulty is to simplify the model. It is proposed here to ignore all the exchanges between the internal and external environments, with the exception of oxygen and carbon dioxide. Fortunately, there is a compelling rationale for this radical simplification.

It deserves to be better known that, during winter, bears enter into a period of "pseudo-hibernation". In this state, the bears, unlike true or "deep" hibernators, allow their body temperature to drop only a few degrees (from 37–39°C to 31–35°C). For a period varying from 3 to 5 months the bears neither eat nor drink. Equally remarkable, the animals are said not to urinate or defecate at any time during their "pseudo-hibernation" (see Brown *et al.*, 1971; Nelson *et al.*, 1973; Nelson, 1980).

It may be objected that this behavior appears to be altogether unique among mammals. While this is possibly true, it is quite doubtful that there is anything structurally unique to bears which accounts for this behavior (see also Britton and Silvette, 1937). More probably, other mammals could be induced to behave in the same way by subjecting them to an endocrine regime comparable to that found in bears during "pseudo-hibernation" (e.g. see Nelson *et al.*, 1973, p. 496).

Again, it might be objected that during this period of "pseudo-hibernation" the animals are not in a steady-state. While true, the force of this objection is limited. It is likely that no living mammal would be found, strictly speaking, to be in a steady-state for more than a few hours, if a sufficient number of variables could be assayed with sufficient accuracy (consider the progressive character of aging, once maturity is reached). Thus, the quasi-steady-state achieved by bears during their "pseudo-hibernation" is likely to be a satisfactory approximation from a model-building standpoint.

Even with this simplification the building of a scalable model of energy partitioning in mammals presents a considerable challenge. The role of the empirical regression equations (e.g. see Tables 1 and 2) may be chiefly to place constraints on the values of the parameters appearing in the physically based equations. For example, in the case of the kidney, the regression equations for the number and diameter of the glomeruli, combined with assumptions as to capillary diameter, can be used to place upper and lower bounds on the surface area available for ultrafiltration. Once a sufficient number of constraints can be

imposed, it may be possible to solve the model equations using the technique of linear programming (e.g. see Jelen, 1970).

At the same time, the construction of scalable models for the individual organs is an important task, complementary to that described above. Such a model, for the mammalian kidney, has been presented by Holt and Rhode (1976). See also Knepper *et al.* (1976) for a model which is relevant to this objective.

SUMMARY

Kidney weight in adult terrestrial mammals varies as the 0.85 power of body weight. Kidney weight varies as the 0.92 power of body weight in aquatic mammals. In both terrestrial and aquatic mammals the slopes of the regression lines are independent of sex. Renal blood flow varies as the 0.82 power of body weight ($pWR = 3.4$). On present evidence, renal blood flow in different mammals represents, to a first approximation, a constant fraction or cardiac output.

The number of glomeruli and the mean glomerular diameter were found to vary as the 0.59 and 0.11 powers of body weight, respectively.

A compartment model, with fluxes between the internal and external environments is introduced, as a first step towards integrating organ-specific energy metabolism into a comprehensive scheme. It is suggested that there are plausible grounds for ignoring the nonrespiratory fluxes, at least in the initial implementation of the model. It is argued that the construction of such a model is an essential prerequisite to an understanding of energy metabolism in mammals.

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AMMONIA ABSORPTION FROM THE INTESTINE IN ADULT COCKERELS

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Abstract—1. Two ml of 2 mM ammonium acetate solution was introduced into 10 cm chicken intestinal sack having Meckel's diverticulum in the middle part.

2. After the introduction, blood ammonia concentration in the mesenteric vein draining the sack reached a maximal increase within 5 min and returned to the initial level in 30 min. The difference of ammonia concentration between cardiac and mesenteric venous blood also showed a similar tendency.

3. About 91% of the ammonia introduced into the intestinal lumen disappeared in 30 min.

4. Amino acid metabolism in intestinal tissue appears to be affected by the introduction of ammonia into the intestinal lumen.

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INTRODUCTION

Large quantities of ammonia are normally present in the portal blood of various animals. Parnas and Klisiecki (1926) with the rabbit found a much higher level of blood ammonia in the vein draining the lower small intestine, caecum and upper large intestine than in the peripheral blood. Cholopoff (1927) obtained similar results with the dog. In the chicken, blood ammonia concentration is also higher in the portal vein than in the heart (Karasawa, 1981).

According to Phear and Reubner (1956), the common intestinal bacteria except possibly some lactobacilli produce ammonia *in vitro*. Reubner and McLaren (1958) showed that the elimination of Bacteroides group by oxytetracycline in caecal content appreciably lowered the ammonia level in the caecal mesenteric vein. These facts suggest that ammonia in the portal blood comes from ammonia production by intestinal organisms. However, there is no direct demonstration about ammonia absorption from the intestine in any animals. The present *in situ* experiment was carried out to examine ammonia absorption from chicken intestine and to examine amino acid metabolism in the intestinal tissue during the ammonia absorption.

MATERIALS AND METHODS

Animals and diet

Experimental animals used in this experiment were adult dwarf-type Single Comb White Leghorn chickens (*Gallus domesticus*), weighing about 1.4 kg, which were obtained from Nagano Animal Industry Experiment Station (Nagano-ken, Japan). The chickens were housed in individual cages in a light-controlled room (12 hr light : 12 hr dark), were fed a commercial diet (CP 14%, ME 2700 Cal: Nippon Haigo Shiryo Co., Tokyo, Japan) *ad libitum* and were allowed to drink water freely. Before the absorption study the birds were allowed only water during 30 hr.

Experimental procedure

Cardiac catheterization was performed in the chickens anesthetized by the intravenous injection of sodium pentobarbital (25 mg/kg body wt), as previously reported (Karasawa *et al.*, 1973). This catheter was used for collecting cardiac blood samples. Then, an abdominal incision was made, the intestine was drawn out, and the small intestine with the mesenteric vein draining it and with Meckel's diverticulum in the middle part was cut off 12 cm long. After the lumen of the segment was washed out with 10 ml of warm distilled water (39°C) by glass pipette to exclude intestinal contents, both sides of the segment were ligated with silk to make a sack (10 cm long). The segment was kept warm throughout the experiment by saline packs. The mesenteric vein draining the sack was cannulated by a small polyethylene tubing (Hibiki No. 4) through which the blood was collected. After the preparation of the intestinal segment and the cannulation of its vein, resting blood samples were collected through cardiac catheter and mesenteric cannula and then 3 ml of 2 mM ammonium acetate solution (pH 6.3) or distilled water (control), warmed to 39°C, was introduced into the lumen with syringe. For analysis of initial lumen ammonia content one ml of the introduced lumen fluid was withdrawn just after finishing the introduction. Cardiac blood was collected at 10 and 30 min and mesenteric blood at 5, 10 and 30 min after the introduction of ammonia into the segment. At the end of experiment the introduced lumen fluid was recovered to determine remaining ammonia.

Chemical analysis

Intestinal mucosa was scraped off tested intestinal segment with spatula, and homogenized with teflon pestle. On the homogenate and cardiac and mesenteric blood, glutamine, glutamic acid, alanine and amino nitrogen were determined. Ammonia was analyzed by the colorimetric method (Okuda and Fujii, 1966), and glutamine by the method described by Goldstein (1966). Glutamic acid was determined with glutamic dehydrogenase by the method of Bernt and Bergmeyer (1963) and alanine by the colorimetric method of Alexander and Seligman (1945) as modified by Christensen *et al.* (1952). Amino nitrogen was estimated by the DNP method of Rapp (1963).

RESULTS AND DISCUSSION

Figure 1 shows time course changes in blood ammonia concentration in the mesenteric vein draining the intestinal sack into which ammonia was

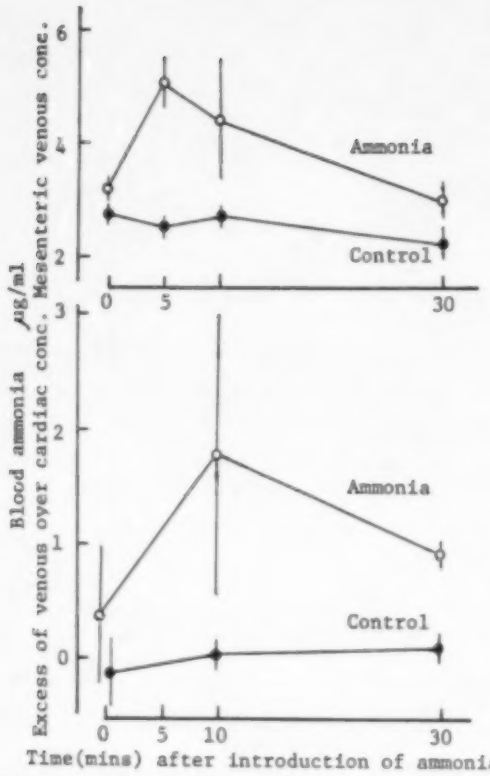

Fig. 1. Blood ammonia concentration in the mesenteric vein and the difference in blood ammonia concentration between mesenteric vein and heart after introduction of ammonia into lumen of experimental portion of intestine. Each point represents mean ± SEM of 3 chickens.

introduced. The ammonia concentration reached a maximal increase within 5 min and returned to the initial level in 30 min. These changes may show not only the increases due to the introduction of ammonia into intestinal lumen but also the secondary increase due to an augmentation in arterial blood ammonia. However, the difference in blood ammonia concentration between mesenteric vein and heart, an indication of net changes due to the introduction of ammonia into the intestinal lumen, showed a similar changing pattern to the ammonia concentration in the mesenteric venous blood (Fig. 1). These results indicate that ammonia in the portal blood rapidly increase when ammonia was produced and increased in the intestinal lumen. Table 1 showed that more than 91% of the introduced ammonia disappeared from the intestinal lumen in 30 min. The large disappearance of the introduced ammonia suggest most ammonia increase in the mesenteric blood to be derived from the introduced ammonia.

According to Phear and Reubner (1956), the common intestinal bacteria except possibly some lactobacilli produce ammonia *in vitro*. With the rabbit blood ammonia concentration in the vein draining the lower small intestine, caecum and upper large intestine is shown to be much higher than in peripheral blood (Parnas and Klisiecki, 1926). Similar results are reported in the dog (Cholopoff, 1927) and chicken (Karasawa, 1981). Moreover, the elimination of bacteroides group by oxytetracycline in caecal content lowers the ammonia level in the caecal mesenteric vein (Reubner and McLaren, 1958). These facts suggest that ammonia in the portal blood comes from ammonia produced in the intestinal lumen by intestinal bacteria. This suggestion was supported by the data mentioned above.

Neam and Wiseman (1957) reported for dogs that glutamic and aspartic acids were transaminated in the intestinal tissue during the absorption processes and there was a resultant increase of alanine in the mesenteric blood. In chickens alanine and serine in the mesenteric blood are also increased when an amino acid mixture composed of 18 kinds of amino acids was introduced into the small intestine (Yokota and Tasaki, 1974). In addition chicken intestine has an appreciable activity of glutamine synthetase involved in glutamine formation from glutamic acid and ammonia (Karasawa and Tasaki, 1974). From these facts there is the possibility that lumen ammonia affects amino acid metabolism in the intestinal tissue, and in consequence the amino acid levels in the mesenteric blood. As shown in Table 2 the introduction of ammonia into the intestinal lumen resulted in significant increases in glutamine ($P < 0.01$) and amino nitrogen ($P < 0.05$) content in the intestinal tissue. Ammonia and glutamic acid content in the tissue also tend to increase and decrease respectively, although not significantly, whereas intestinal tissue

Table 1. Disappearance of introduced ammonia from intestinal lumen fluid in 30 min

Introduction into lumen	Lumen ammonia Initial	Lumen ammonia Final (μg/tested portion)	Disappearance of ammonia	Disappearance of ammonia (% of introduced amount)
Control	1.47 ± 0.29	2.01 ± 0.25	−0.54 ± 0.17	
Ammonia	73.12 ± 11.82	6.16 ± 1.97	67.00 ± 8.97	91.47 ± 2.22

Values are means ± SEM of 3 chickens.

Table 2. Amino acid contents in intestinal mucosa 30 min after introduction of ammonia into lumen of experimental portion of intestine

Introduction into lumen	Glutamic acid (μg/g wet wt)	Glutamine (mg/g wet wt)	Alanine (μg/g wet wt)	Amino-N (mg/g wet wt)
Control	100.0 ± 14.9	0.49 ± 0.03	117.5 ± 14.0	0.59 ± 0.11
Ammonia	63.7 ± 2.6	2.25 ± 0.25**	142.3 ± 37.1	1.23 ± 0.03*

*, ** Significantly different from each control value at $P < 0.05$ and $P < 0.01$, respectively. Values are means ± SEM of 3 chickens.

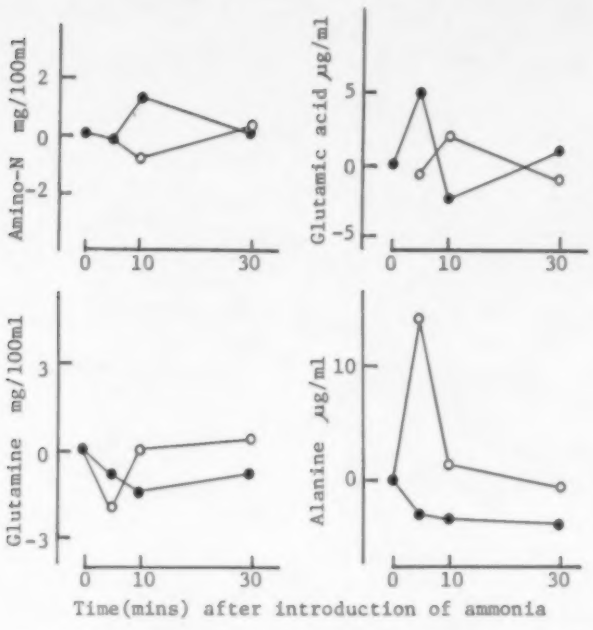

Fig. 2. Amino nitrogen, glutamic acid, glutamine and alanine concentrations in the mesenteric blood after introduction of ammonia into lumen of experimental portion of intestine. ○, Ammonia introduced; ●, control. Each point represents mean of 3 chickens.

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alanine was not affected by ammonia in the lumen. On the other hand, blood alanine concentration in the mesenteric vein showed significant increase 5 min after the introduction of ammonia into the intestinal lumen ($P < 0.05$) but no change in amino nitrogen, glutamic acid and glutamine in the mesenteric venous blood (Fig. 2). These results indicate that amino acid metabolism in the intestinal tissue is affected by an increase in intestinal lumen ammonia.

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PATTERNS OF PERIPHERAL VASOMOTOR FUNCTION IN NEAR-NATURAL CONDITIONS IN THE CALF (*BOS TAURUS*)

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Abstract—1. Patterns of ear temperatures were examined in 2- to 45-day-old calves.

2. At variable heat loads, peripheral temperature levels varied with the heat load with little fluctuations.

3. When 2 to 3 days old, high ear temperatures were maintained for 10–14 hr/day at 10–15°C ambient temperatures in 5 of 8 calves.

4. In near-natural conditions, fluctuating ear temperatures were the rule in the vasodilated, intermediate and vasoconstricted states; fluctuations were symmetrical in the two ears in the vasodilated and intermediate states only.

5. The possible occurrence of cold-induced vasodilation at 10–20°C ambient temperature is discussed.

INTRODUCTION

The temperature of the surface of the body is sensitive to changes in the ambient temperature and in the rate of blood flow. The surface of the body extremities is particularly sensitive to these changes, due to their large surface-to-mass ratio. The rate of blood flow in the periphery of the body is modulated by the action of the sympathetic system on the resistance vessels as well as on the arteriovenous anastomoses. Alterations in the surface temperature of body extremities are part of the control of body temperature, modifying the rate of heat loss to the environment. A cold stimulus reduces blood flow to body extremities resulting in a decreased loss of heat to the environment (Iriki *et al.*, 1971).

At low ambient temperatures, close to freezing, a hunting reflex appears in body extremities; it is characterized by repeated increases in blood flow and skin temperature (Lewis, 1930; Edwards and Burton, 1960). These sudden increases in temperature usually appear after periods of cold-induced vasoconstriction. The hunting reflex has been shown in man (Lewis, 1930), fowl (Grant and Bald, 1932), rat (Hellstrom, 1975) and sheep (Meyer and Webster, 1971). The hunting reflex, in contrast with cutaneous sympathetic vasomotor tone, appears to be controlled mainly by local factors (Vanhoutte, 1980), and therefore it entails a certain temporary loss of the thermoregulatory function of the peripheral organs.

These two types of temperature changes in extremities have been demonstrated mostly in laboratory conditions and in mature animals. This study examined the patterns of the temperature changes in body extremities of the neonate calf and their alteration with age, in the less extreme near-natural conditions in which calves are normally reared.

MATERIALS AND METHODS

Animals

The study has been carried out in two separate experiments. Both experiments were performed on Israeli–Holstein calves of either sex. Experiments began at the age of 2 days, following the completion of colostrum feeding. The calves were kept in individual pens (0.7 × 1.4 m) liberally bedded with straw. The pens were located in an enclosed barn, in which air movement was low (0.1–0.3 m/sec), and air temperature equalled the outdoor temperature. The animals were fed twice a day (at 0600–0700 hr and at 1600–1800 hr) a commercial milk replacer diluted to 125 g/l.; they were given 3 l./day in the first week, 4 l./day in the second week, 5 l./day in the 3rd week and 6 l./day in the 4th week. A starter mixture and hay were offered *ad libitum* starting from 2 weeks of age. The animals were generally healthy; the sporadic cases of diarrhoea that occurred were treated and lasted for a few days only. No observations were performed on animals that showed any gatrointestinal disturbances.

Temperature measurements

Body and ear pinna skin temperature were measured by temperature transmitters built according to a variation of a standard design (MacKay, 1970); the transmitters weighed about 30–40 g including the battery and the silastic coating. The body temperature transmitter was built as one single-coated unit. The ear temperature transmitter was modified by separating the temperature-sensitive thermistor from the emitter and attaching it to the ear pinna using a cloth peg, the spring of which was loosened so that it would not press the ear tissues. The ear transmitters were calibrated for the temperature range of 10–38°C and the body transmitters for the 37–40°C range. The transmitters were calibrated in a thermostatic bath (Model EC-2, Haake, Berlin), the temperature of which was measured to an accuracy of ± 0.1°C using a thermistor probe (Model 403, monitor model 46-TUC, YSI). The signals of the transmitters were received by commercial FM receivers and the time intervals were determined on timer-counters (Counter Model DP-150A, Hickok). During experiments the signals were recorded on tape recorders (Model TC-105, Sony) actuated at preset time intervals by a timer, and read later on the timer-counters. A computer program was used to translate the time intervals of the signals into temperature data, to plot the resulting graphs, as well as to analyze the data. The temperature transmitters were periodically examined for possible shifts in their calibration curves. The body temperature transmitters were administered by bolus gun to 6 animals, in which they were retained in the lower rumen. To

Table 1. Number of animals observed, mean (±SE) number of 24-hr periods observed per animal and ambient temperatures at each week of age

	Age (weeks)				
	1	2	3	4	5
No. of animals examined	8	7	8	7	6
No. of 24-hr periods/animal.	4.9 ± 0.3	1.6 ± 0.2	2.5 ± 0.4	2.9 ± 0.6	1.7 ± 0.2
Weekly mean air temperature (°C)	15.5 ± 1.0	15.9 ± 1.5	15.3 ± 1.4	15.6 ± 1.1	15.7 ± 2.6
Weekly mean maximum air temperature (°C)	20.1 ± 1.3	20.9 ± 1.7	20.7 ± 2.4	19.8 ± 1.1	21.1 ± 2.8
Weekly mean minimum air temperature (°C)	12.0 ± 0.9	12.2 ± 1.2	11.3 ± 1.1	12.6 ± 1.2	10.7 ± 2.3

examine for shifts in their calibration, rectal temperatures were measured twice daily by thermistor probes (Model 427, YSI) accurate to ±0.1°C; these probes were inserted into the rectum to a depth of 12 cm. The body and ear temperatures were measured at weekly intervals, each week for at least one 24-hr period. Ambient air temperatures and humidity were continuously recorded using a calibrated thermohygrograph (Thies, Gottingen).

In addition to the measurement of the spontaneous changes in ear and body temperatures occurring during the 24-hr cycle, the ambient conditions were varied on three of the animals. This was performed by either increasing the radiant heat load on the hind part of the animals by a brooder hanging above the animal, or by setting a fan behind the animal. In both cases, the ears and the head of the animals were shielded by a 1-inch-thick polystyrene plate set on the neck. In these experiments, rectal, shank and back skin temperature were also measured. For the latter, small specially prepared thermistor probes were used, attached to the skin by a rubber base cement. Also, combined radiant and convective heat loss from the rump was determined using a Hatfield disc.

RESULTS

Body temperature and ear pinna temperatures (Te) were to be examined for at least one complete 24-hr cycle on each week of age in the 9 calves in the experiment. Technical problems prevented this in a number of cases. Details on the number of animals observed, the number of 24-hr cycles measured, and ambient temperatures prevailing on each week of age are presented in Table 1.

The number of 24-hr observation periods measured was greater in the first week of age, to examine in more detail possible postnatal changes in responses. Ambient temperatures were similar at all ages, with relatively small variation between animals, as the study was carried out in about the same season on two successive years. This makes possible a comparison of responses at different ages, without this being obscured by differences in ambient conditions.

Symmetry of Te changes in the two ears

The extent to which Te changed symmetrically in both ears, was evaluated by correlations between concomitant left ear and right ear temperatures. These were calculated separately for each week of age, to examine the effect of age on the symmetry of response. Also calculated were the correlations between right and left ear temperatures and between air temperatures. These data are presented in Table 2.

The correlation between right and left ear temperatures was statistically significant at all ages ($P < 0.01$); a certain increase in the relationship was evident, mostly from the age of 3 weeks. The relationship between ear temperatures and air temperatures was similar in both ears, and was not affected by age. A more detailed visual examination of the individual 24-hr groups indicated that the relationship between the temperature of the two ears was closer during the prevalence of a sustained vasodilation (Fig. 1); as long as this state prevailed, the temperatures of the two ears were close to each other, and the changes occurring were generally symmetrical.

Patterns of ear temperature

A visual detailed assessment of the individual graphs, each representing a 24-hr period, indicated that ear pinna skin temperatures could be sorted into three main patterns:

(a) Te of about 30°C (indicative of a vasodilated state at these ambient temperatures), with small parallel fluctuations in the two ears which were maintained over a few hours in both ears (Fig. 2);

(b) Te fluctuations of moderate amplitude with a general trend of increase or decrease of the mean Te which occurred concurrently in the two ears (Fig. 3);

(c) large fluctuations in ear temperature, ranging from close to air temperature to levels of about 30°C which were separated by extended periods of Te close to air temperature, indicative of a state of vaso-

Table 2. Relationship between left and right ear temperatures and between ear temperature and air temperature at 1 to 5 weeks of age

		Age (weeks)				
		1	2	3	4	5
Parameter						
Left ear/right ear	r	0.68	0.66	0.78	0.88	0.80
	R^2	0.47	0.44	0.61	0.78	0.64
	n	2644	852	1665	1664	896
Left ear/air temperature	r	0.64	0.60	0.67	0.69	0.65
	R^2	0.41	0.35	0.44	0.47	0.42
	n	2690	926	1651	1764	902
Right ear/air temperature	r	0.61	0.56	0.68	0.70	0.60
	R^2	0.37	0.32	0.46	0.49	0.36
	n	2884	928	1745	1656	889

n, Number of observations.

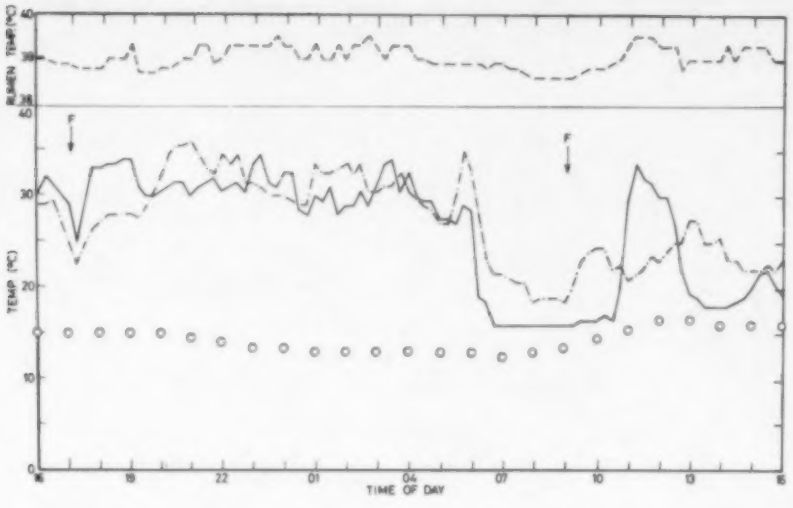

Fig. 1. Ear pinnae and rumen temperature changes during a 24-hr cycle. Calf No. 4 at the age of 2 days. Meal offered at times marked by F; food otherwise withheld. Rumen temperatures ---. Ear pinnae temperatures: left ear —·—, right ear ——. Air temperatures, ○.

constriction, lasting for up to a few hours. These fluctuations were, as a rule, asynchronous in the two ears, although a few exceptions were found. A typical case is shown in (Fig. 1).

Effect of age on Te patterns

On examining the Te data of the animals in their first week of life, it became evident that the animals could be classified into two groups on the basis of their responses in the first 2 to 3 days of life. In the first group, represented by 5 animals (numbers 1, 2, 4, 5 and 8), a sustained vasodilation prevailed for 10–14 hr of the day, even at relatively low ambient temperatures. The sustained vasodilation was followed by a prolonged vasoconstriction lasting for a few hours. These two events occurred at similar air temperatures, 15.4 ± 1.5°C and 13.5 ± 0.7°C. A typical response of such an animal is shown in Fig. 2.

In the other 4 animals, as well as in the animals of the first group in the latter days of the first week of life, a fluctuating response was typical, in which prolonged periods of vasoconstriction were evident, lasting for 5–6 hr (Fig. 3).

As previously mentioned, a fluctuating response of Te was typical in the latter days of the first week of life. It remained the characteristic response in the subsequent weeks, up to week 5 inclusive, at which time the observations were terminated.

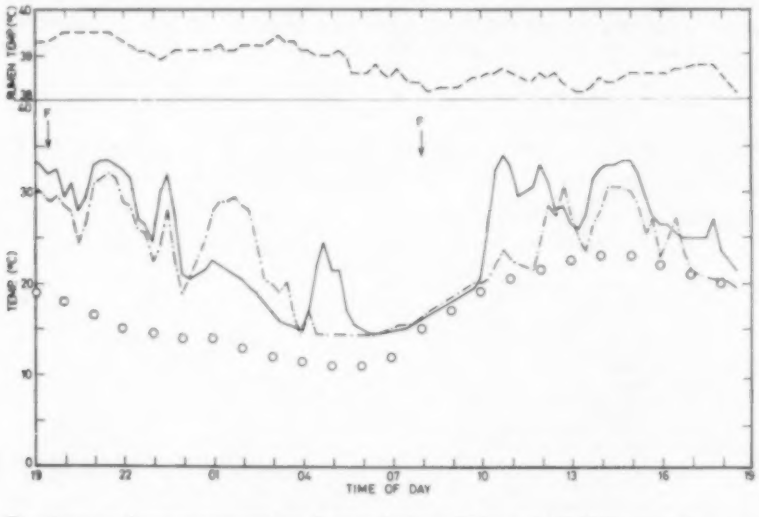

Fig. 2. Ear pinnae and rumen temperature changes during a 24-hr cycle. Calf No. 7, at the age of 4 days. Legend as in Fig. 1.

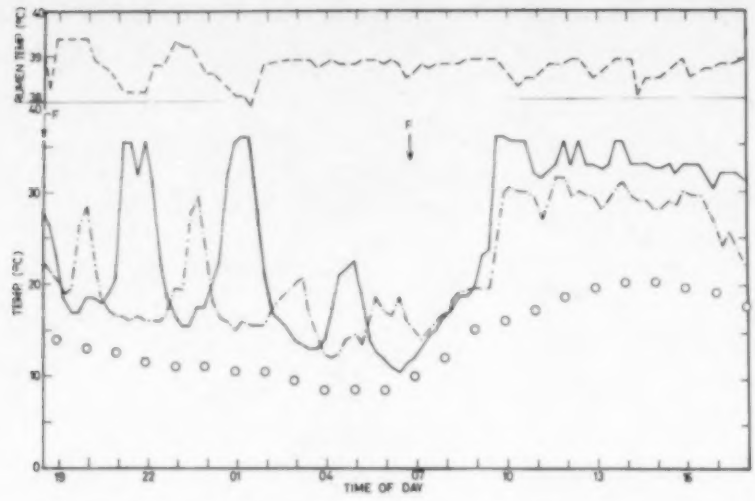

Fig. 3. Ear pinnae and rumen temperature changes during a 24-hr cycle. Calf No. 2, at the age of 22 days. Legend as in Fig. 1.

Effects of feeding

In all animals, and at all ages, feeding the morning milk ration was followed by an increase in Te (Figs 2 and 3). The time lapsed between the feeding and the increase in Te was negatively correlated with ambient temperature at the time of feeding. The regression between the ambient temperature (Ta) and time lapsed (t) between feeding and the increase in Te was $b = 354 - 20.3 \cdot \mathrm{Ta}$; $r = 0.766$; $P < 0.01$. The increase in Te was not consistently associated with a particular change in intraruminal temperature; a concomitant increase in the latter was observed as frequently as a concomitant decrease in it.

It should be, however, noted that such an increase in Te following feeding was not observed when the other half of the daily ration was fed at 18 hr. This might be possibly related to the consistent prevalence of a prolonged ear vasoconstriction in the hours preceding the morning feeding, but not the afternoon feeding. This prolonged vasoconstriction in the morning was a typical response in almost all the observations.

Effect of age on the prevailing vasomotor state

The individual graphs were examined for the prevalence of the three main vasomotor states, namely prolonged vasoconstriction, fluctuating response and sustained vasodilation. It was observed that the daily duration of these vasomotor states changed with both age and the ambient temperature. To demonstrate the patterns of these changes, the data of 4 typical animals are presented in Table 3. The data were selected on the basis for their representing animals exposed to different ambient temperatures, mean values ranging from 13 to 24.3°C.

The overall trend of the data was of a gradual increase with age in the relative duration of the sustained vasodilated state during the 24-hr cycle. The increase in the prevalence of vasodilation occurred by a gradual reduction in the duration of

Table 3. Change with age in duration of each vasomotor state, expressed as percentage of 24 hr at four ambient temperatures, in 4 out of 9 calves

		Animal			
Age (weeks)	Vasomotor response	A	B	C	D
1	Vasodilated	37	18	19	55
	Fluctuating	52	57	61	9
	Vasoconstricted	11	25	21	36
2	Vasodilated		—	53	70
	Fluctuating		67	15	30
	Vasoconstricted		33	32	—
3	Vasodilated	2	21	70	100
	Fluctuating	53	59	30	—
	Vasoconstricted	45	20	—	—
4	Vasodilated	18	95	100	98
	Fluctuating	30	—	—	—
	Vasoconstricted	52	5	—	2
5	Vasodilated	—	93	100	98
	Fluctuating	81	2	—	—
	Vasoconstricted	19	5	—	2
Mean (±SE) daily temperature (°C)		13.0 ± 1.4	16.2 ± 1.4	21.3 ± 2.0	24.3 ± 0.1

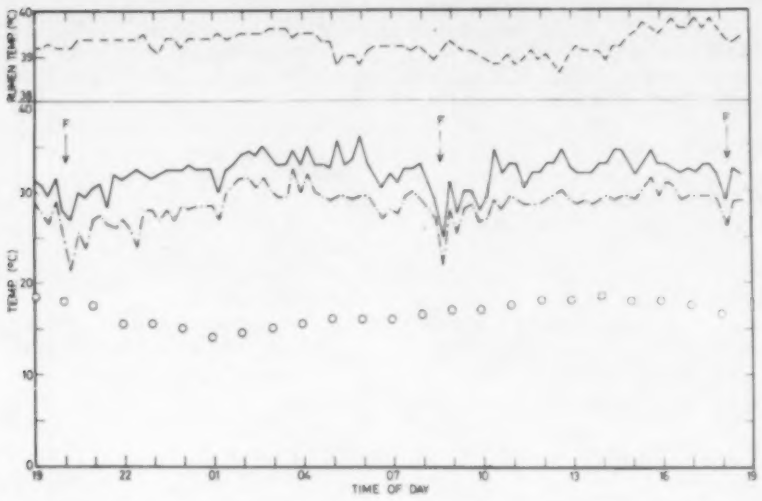

Fig. 4. Ear pinnae and rumen temperature changes during a 24-hr cycle. Calf No. 3, at the age of 22 days. Legend as in Fig. 1.

the vasoconstricted state, concomitant with an increase in the duration of the fluctuating response state. At later ages, continuously vasodilated ears were typical (Fig. 4).

Variable heat load effects

The earlier experiments examined the responses when the animals were exposed to the relatively stable heat load under the natural rate of change in air temperature (1–1.5°C/hr); to examine the responses occurring on a step change in heat load, a step change in heat load was created by either imposing a radiant heat load on the hind part of the animal or by enhancing heat loss using forced ventilation. These were performed while the animal was standing in the pen. A total of 10 such experiments were carried out on 3 animals, over an age span of 8–28 days. A typical response in such an experiment is shown in Fig. 5.

The responses to forced ventilation were of a gradual reduction in ear and shank temperatures over the 80 min of such a treatment, until they reached levels close to air temperature. During the 100 min following the cessation of the forced ventilation peripheral temperatures remained stable, as in the case shown in Fig. 5, or gradually increased to the

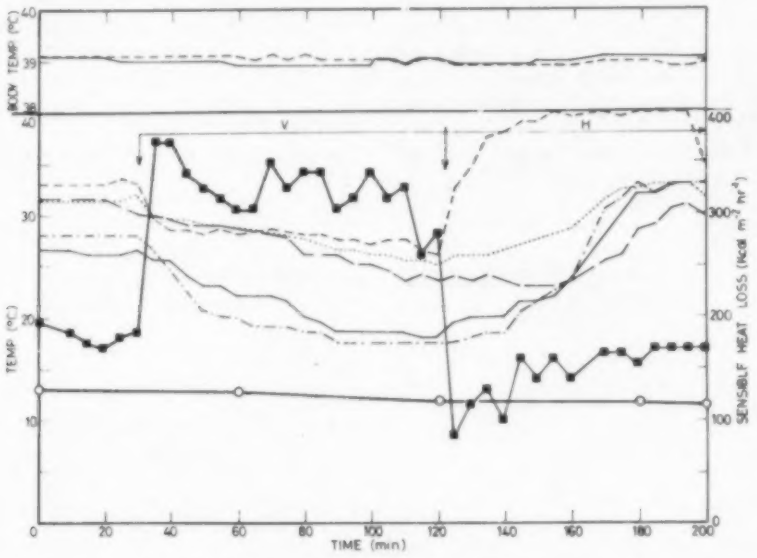

Fig. 5. Ear pinnae, shanks and rumen temperatures and sensible heat loss during forced ventilation and radiant heat load. Calf No. 11 at the age of 25 days. Rectal temperature ——, rumen temperature – – –. Shank skin temperatures: left shank – – –; right shank ——. Sensible heat loss, ■. V, time during which forced ventilation was applied. H, time during which radiant heat was applied.

pretreatment level. The opposite trend of change was observed on the imposition of a radiant heat load. In most of the experiments the changes in peripheral temperatures were synchronous in both ears and legs. No effect of age on the pattern of response was noted.

Body temperature

The rectal temperatures of the animals were measured twice daily before feeding, at 0600–0700 hr and at 1600–1800 hr, by using thermistor probes. The mean values for each week of age are presented in Table 4.

A diurnal 0.4°C increment in rectal temperature from morning to evening was evident. This increment did not change with age. Also, a trend for an increase in the level of body temperature with age, starting at 3 weeks of age, was observed. This is probably related to the onset of ruminal function, since it was not associated with a significant increase in air temperature.

DISCUSSION

The present study indicated that the ear temperatures of the calf were of a fluctuating nature. These fluctuations were smaller when ear temperatures were high, i.e. in a vasodilated state. The fluctuations were large in amplitude when the baseline level was close to the ambient temperature, and of moderate amplitude at intermediate levels of the baseline ear temperature.

These results are in contrast to reports on ear skin temperature responses to variable air temperatures in the calf and rabbit (Gonzalez-Jiminez and Blaxter, 1962; Harada and Kanno, 1975). In those latter studies, the typical response to an increase in air temperature was a gradual increase in ear temperature, forming an asymptotic, S-shaped curve of the ear temperature response. A similar response was only observed, in our study, following feeding or on a step change in heat loss or in heat load. It seems, therefore, probable that the fluctuating response in the sheltered, near-natural environment is related to the slow and gradual changes occurring in the air temperature. These changes usually are of 1–1.5°C/hr. Such fluctuating ear temperatures were indeed reported in animals kept at moderate constant air temperatures: in cats (Adams *et al.*, 1970), rabbits (Harada and Kano, 1975), and calves (Whittow, 1962).

Behavioral changes in posture, however, also possibly affect the pattern of ear temperature changes. A prolonged vasoconstriction was common in the morning hours preceding feeding. At this time, the typical recumbent posture in which the calf spends most of the night changes to a more restless, standing posture. This posture change is associated with a sudden increase in heat loss. The prolonged vasoconstriction might thus be associated with this sudden increase in heat loss, as also occurred when the animals were subjected to a sudden increase in heat loss, caused by forced ventilation. It seems, therefore, that the two patterns of ear temperature responses might appear, depending on the rate of change in ambient conditions.

As was mentioned in the Results section, the fluctuations in ear temperature could be classified into three different patterns. The first was of fluctuations of a few degrees in amplitude, symmetrical in both ears, which occurred when ear temperatures were in the 30°C range. The second was of wider symmetrical fluctuations, with a 5–10°C amplitude, occurring when mean ear temperatures were either increasing or decreasing. These two patterns probably reflect different degrees of the thermoregulatory control over sympathetic vasomotor function. Alterations in the vasomotor tone were shown to cause shifts in peripheral temperature (Adams *et al.*, 1970). As both static and transient temperatures may provide temperature information to the thermoregulatory system (Pierau and Wurster, 1981), the two patterns of ear temperature changes may indicate a higher intensity of sympathetic efferent output during transient heat loads.

In the third pattern, changes in ear temperature were asynchronous in the two ears. It is thus probably of a different nature. It resembles the cold-induced vasodilation (CIVD) first described by Lewis (1932), in the amplitude of the changes, in their being asynchronous in the two ears, and in that the increases in ear temperature occurred from a baseline close to air temperature. It differs from the CIVD in two respects. The one is that CIVD was described to occur at ambient temperatures close to 0°C and below it, while in these observations the changes occurred at ambient temperatures of 10–20°C. The other is the repetition of the CIVD at 15- to 45-min intervals (Grant *et al.*, 1933; Edwards and Burton, 1960), while in this experiment the increases in ear temperature took place at much longer intervals, and also lasted for up to 2 hr.

The question thus arises whether this pattern of repeated vasodilations can be assumed to be related to the CIVD. Asynchronous changes in temperature

Table 4. Rectal and air temperatures (°C, ± mean SE), measured in the morning (0600–0700 hr) and in the evening (1600–1800 hr) at 1–5 weeks of age

Parameter	Age (weeks) 1	2	3	4	5
Rectal temperature					
Morning	38.6 ± 0.01	38.6 ± 0.05	38.7 ± 0.03	38.8 ± 0.02	38.9 ± 0.04
n	33	11	22	18	10
Evening	39.0 ± 0.01	39.0 ± 0.04	39.1 ± 0.04	39.1 ± 0.03	39.2 ± 0.05
n	36	13	23	22	7
Air temperature					
Morning	15.9 ± 2.3	16.3 ± 1.7	16.9 ± 2.8	15.7 ± 1.9	16.8 ± 2.3
Evening	19.0 ± 2.5	18.7 ± 2.1	18.3 ± 2.6	19.2 ± 1.5	20.3 ± 2.5

n, Number of observations.

in the two ears can be attributed to local changes which temporarily affect the response of the vascular tissues to the sympathetic tone.

An increase in sympathetic tone, as a result of low ambient temperatures, has the effect of reducing blood flow by acting on the precapillary smooth muscle rings and on the glomi of the arteriovenous anastomoses. The result is a gradual fall in the temperature of a peripheral organ like the ear. Cooling adrenergic nerves to 15°C or below depressed their function (Potter, 1966). The responsiveness of blood vessels in the perfused ear of the rabbit, sheep, pig and ox to vasoconstrictor agonists increased with a reduction in temperature to about 20°C; a further reduction in temperature caused decreased responsiveness until practically no response was observed at about 10°C (Martin and Wallace, 1970; Surgeon and Wallace, 1975). In addition to these factors a reduced flow of blood in an organ would also increase the partial pressure of CO_2 and reduce that of O_2, both factors known to reduce vascular resistance (Daugherty *et al.*, 1967). In the sympathetic denervated ear vasodilation was present in the cold (Meyer and Webster, 1971). The concurrence of these factors would thus probably create the repetitive nature of a CIVD, by temporarily overriding the constrictor effect of the sympathetic tone even at ambient temperatures ranging between 10 and 20°C.

It seems, therefore, possible that when the cold stimulus is lighter and the sympathetic induced constrictor drive is lesser, a more prolonged vasoconstriction such as observed here would be possible, as long as ear temperatures would stay at levels which do not inhibit vasoconstriction; this might be the basis for the sustained vasoconstriction at the more moderate temperatures at which these observations were carried out.

SUMMARY

Peripheral temperatures were continuously monitored in calves aged 2–45 days, exposed either to the slow ambient temperatures of a near natural environment or to heat load transients. When exposed to transient heat loads, as induced by forced ventilation or radiant heat, as well as after feeding, the level of peripheral temperatures changed gradually to a new level, with little if any fluctuations. When exposed to the near natural conditions (10–20°C) fluctuating ear temperatures were the rule. For the first 2–3 days of life, a sustained ear vasodilation prevailed for 10–14 hr of the day in 5 out of 8 animals. The incidence of vasodilation increased with age, to become predominant at 5 weeks of age. The fluctuations in ear temperatures were symmetrical in the two ears when these occurred in the vasodilated and intermediate states, but not in the vasoconstricted state. The possibility that the fluctuations occurring in the vasoconstricted state, at 10–15°C ambient temperature are similar to the cold-induced vasodilation is discussed.

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CARBON DIOXIDE PRODUCTION BY RED SKELETAL MUSCLE OF GOLDFISH (*CARASSIUS AURATUS* L.) AEROBIC AND ANAEROBIC METABOLISM OF GLUCOSE AND GLUTAMATE

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(*Received* 24 *March* 1983)

Abstract—1. Oxygen uptake and metabolic CO_2 production by lateral red muscle of goldfish have been measured *in vitro*.

2. Added glucose 6-phosphate depresses the rate of oxygen uptake by minced red muscle (Crabtree effect). Total CO_2 production is stimulated resulting in a respiratory quotient which is considerably greater than one.

3. $^{14}CO_2$ release from [U-^{14}C] glucose 6-phosphate and [U-^{14}C] glutamate continues during anoxia.

4. No activity of the hexose monophosphate shunt was observed.

5. The results suggest that both aerobic and anaerobic CO_2 production is of mitochondrial origin and, at least partially, derived from TCA cycle reactions.

INTRODUCTION

Production of metabolic CO_2 may be conceived as an expression of oxidative reactions. In the goldfish (*Carassius auratus* L.) and the crucian carp (*Carassius carassius* L.) the excretion of this fully oxidized compound is a common parameter of aerobic and anaerobic catabolism (Blažka, 1958; Van den Thillart, 1977). However the mechanism underlying the aerobic and anaerobic CO_2 production may be quite different since metabolic pathways will be significantly altered by anoxia.

In skeletal muscle of goldfish part of the anaerobic CO_2 production is linked to alcoholic fermentation (Shoubridge and Hochachka, 1980). The mechanism is based on a cooperation of mitochondrial and cytoplasmic enzymes. Acetaldehyde and CO_2 are released from the pyruvate dehydrogenase complex in red muscle mitochondria. In the cytoplasm a high activity of alcohol dehydrogenase allows the formation of ethanol (Mourik *et al.*, 1982). Since the rate of anaerobic acetaldehyde production is only 30% the rate of pyruvate uptake, a more complex participation of red muscle mitochondria in anaerobic metabolism may be suggested. Experimental data, obtained from anoxic goldfish *in vivo* equally point to the involvement of mitochondria in anaerobic metabolism. From radiorespirometric measurements it was concluded by several authors that the TCA cycle is operational in the anoxic goldfish, although probably at a low rate. Anoxic goldfish at 15°C produce $^{14}CO_2$ from [1-^{14}C] acetate (Hochachka, 1961). No change in the rate of $^{14}CO_2$ production was observed when anoxic goldfish returned to oxygenated water. $^{14}CO_2$ production from [1-^{14}C] acetate by anoxic goldfish at 20°C was substantiated by Van den Thillart and Verbeek (1982). In contrast, Shoubridge (1980) observed virtually no anaerobic $^{14}CO_2$ production from [U-^{14}C] acetate by goldfish at 4°C. So, it seems that at least at higher temperatures part of the anaerobic CO_2 production by goldfish is linked to TCA cycle activity.

In the present study aerobic and anaerobic oxidative metabolism by goldfish red muscle were examined *in vitro*. Production rates of total CO_2 and $^{14}CO_2$ from [U-^{14}C] glucose 6-phosphate and [U-^{14}C] glutamate were determined. Glucose 6-phosphate was used to monitor carbohydrate catabolism in minced red muscle. In disrupted cells glucose 6-P easily enters the glycolytic pathway. Dilution with endogenous glucose units is limited, for glucose 6-P is a strong inhibitor of hexokinase activity (Newsholme and Randle, 1961; Rose and O'Connell, 1964; England and Randle, 1967). Glutamate was chosen as a representative of amino acid catabolism, for it is known that proteins are rapidly oxidized by fish (Nagai and Ikeda, 1972). From the rates of NH_3 and CO_2 excretion by goldfish a 50% protein utilization in aerobic metabolism and at least 25% in anaerobic metabolism was calculated by Van den Thillart (1977).

MATERIALS AND METHODS

Animals and tissue preparation

Goldfish (*Carassius auratus* L.) of 100 g mean weight (14–16 cm body length) was acclimated for at least two months to a temperature of 20°C, normal oxygen levels (pO_2 = 130–160 mm Hg) and a 16 hr light period. The fishes were fed daily with Trouvit pellets (Trouw, The Netherlands), 1.5 g/100 g fish. Before decapitation, the fishes were anaesthetized with 100 ppm ethyl *m*-aminobenzoate (MS 222) to prevent metabolic changes during excision of muscle tissue. In a cold room (4°C) both superficial, lateral red muscles were rapidly excised and cooled on ice. Goldfish red muscle was minced into particles of about 1 mm^3 with a precooled tissue slicer, according to Van den Thillart (1977). In accordance to Hess and Brandt (1970) this preparation, consisting of broken cells and intact intracellular particles, such as mitochondria, appeared to be particularly suited for examination of the overall catabolism of goldfish red muscle. Standard amounts of 100 mg homogeneous minced muscle were utilized for incubation.

Incubation procedures

Minced red muscle was incubated at 20°C in a medium of intracellular Na^+/K^+ composition; 35 mM NaCl, 60 mM

Table 1. The rates of oxygen uptake by minced red muscle of goldfish and variations between groups of fish

Group*	Number of fish (*n*)	Oxygen uptake (nmol O_2/min/100 mg wet wt) Endogenous	2.5 mM glucose 6-P + 1 mM glutamate	Ratio†
I (April	6	29.0 ± 12.4	20.9 ± 10.9	0.81 ± 0.12
II (July)	12	73.0 ± 26.4	49.5 ± 27.1	0.70 ± 0.14
III (November)	7	30.7 ± 15.4	26.8 ± 17.9	0.82 ± 0.19

*A group consists of animals supplied together to the laboratory. The month of experimental use, after acclimation to 20°C, is given.

†Ratio of substrate induced and endogenous respiration is given for quantification of the Crabtree effect. The ratio is expressed as the mean ± SD of several experiments.

Minced red muscle (25–50 mg/ml) was incubated at 20°C in standard incubation medium consisting of 35 mM NaCl, 60 mM KCl, 1.2 mM $MgSO_4$–7 H_2O, 1 mM EGTA, 3.3 mM NaH_2PO_4–H_2O and 13.1 mM Na_2HPO_4–2 H_2O (pH 7.4). Initially the medium was air saturated. Polarographic measurements of oxygen uptake were made as described in Materials and Methods. Each value was the mean of at least 3 separate incubations using red muscle of 1 goldfish per experiment. Results are expressed as the mean ± SD of *n* independent experiments.

KCl, 1.2 mM $MgSO_4$–7 H_2O, 1 mM EGTA, 3.3 mM NaH_2PO_4–H_2O and 13.1 mM Na_2HPO_4–2 H_2O (pH 7.4). Oxygen uptake was measured polarographically with a biological oxygen monitor (Yellow Springs Instruments, USA). Carbon dioxide production was measured in 20 ml screw-capped glass vials for liquid scintillation counting. The vials were sealed by a silicon-rubber disc, tightly fitting in the perforated screw cap through which substrates or inhibitors could be injected into the medium. The incubation medium was supplemented with substrates and 100 mg minced red muscle to a final volume of 0.7 ml. The thin layer of the reaction medium permitted a rapid diffusion of atmospheric oxygen in the presence of actively respiring tissue.

$^{14}CO_2$ production was determined after addition of 0.05–0.1 μCi ^{14}C labelled substrates to the reaction medium. Reactions were terminated and $^{14}CO_2$ was liberated by injection of 0.1 ml 5 N H_2SO_4. D-[U-^{14}C] glucose 6-phosphate, D-[1-^{14}C] glucose, D-[6-^{14}C] glucose and L-[U-^{14}C] glutamate were purchased from the Radiochemical Centre (Amersham, England). The $^{14}CO_2$ trapping system was a modification of the system as employed by Levine and Watts (1966). Metabolically produced $^{14}CO_2$ was absorbed for at least 1 hr to 0.15 ml of 2.7 N KOH on a glass-fiber filter (Whatman GF/A) (Buhler, 1962). After absorption, the moist filters were cautiously removed and dropped into counting vials containing 10 ml of xylene, PPO, POPOP scintillation solution (Lumac, Switzerland). The filters were carefully laid down flat on the bottom of the vials and counted in a Nuclear Chicago Isocap or Mark II scintillation analyser. Counting efficiency was determined by use of a calibration plot of $H^{14}CO_3$ standards. The xylene-immiscible organic bases for $^{14}CO_2$ trapping, such as phenethylamine or hyamine hydroxide, caused almost complete inhibition of the oxidizing system in goldfish red muscle. A similar toxic effect was noted earlier by Duncombe and Rising (1969) and Buhler (1962).

Total CO_2

Conditions for determination of total CO_2 production were essentially similar to those for the determination of $^{14}CO_2$ production except for leaving out the ^{14}C labelled substrates and the $^{14}CO_2$ trapping system. Reactions were terminated by injection of 0.1 ml of 0.5 N KOH and cooling to 0°C. Total CO_2 was determined manometrically by means of a pressure transducer according to the method described by De Renzis and Payan (1976). Change of pressure, after liberation of CO_2 by rapid mixing of the reaction medium and 0.05 ml 10 N H_2SO_4, was registered on a strip chart recorder. CO_2 concentration of the reaction medium were calculated from a calibration graph with $NaHCO_3$ standards. Blank values of the incubation medium and of the non-incubated tissue were subtracted to obtain the quantity of metabolically produced CO_2.

Anoxia

Incubation vials for the determination of anaerobic CO_2 production were flushed collectively with nitrogen gas. The vials were evacuated and refilled with nitrogen gas at 0°C for three times in a vacuum desiccator. The oxygen tension of the gas phase was recorded throughout the procedure. Finally the oxygen tension was 0.30 ± 0.14 mmHg. Calculated, according to Henry's law, this corresponds to a maximum concentration of oxygen of 0.55 μM O_2. This is well below the concentration that can be consumed by actively respiring red muscle mitochondria. (4 μM O_2 at 20°C, personal observation.)

RESULTS

Group dependent variations in metabolic activities

Significant variations in metabolic activities of red muscle tissue were observed between several groups of goldfish (Tables 1 and 4). As shown in Table 1 the mean rate of oxygen uptake by minced red muscle significantly differs between animals of group II and those of group I and III. These variations hold equally for endogenous and for substrate induced respiration, resulting in an almost constant ratio. A similar group dependent metabolic effect is noted on the rates of $^{14}CO_2$ production from [U-^{14}C] glucose 6-P (Table 4). Since the ratio between substrate induced and endogenous respiration and the ratio between anaerobic and aerobic $^{14}CO_2$ production are almost equal in all groups, it seems that the group dependent variations are only of a quantitative character. In general the qualitative aspects of oxidative metabolism are comparable for all groups of fish.

Oxygen uptake by minced red muscle

As shown in Table 1 the rate of oxygen consumption by minced red muscle is suppressed to about 75% of

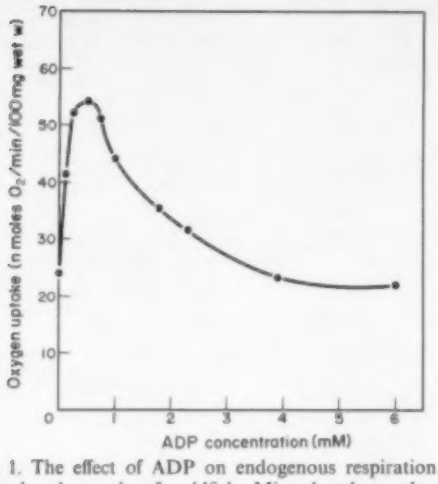

Fig. 1. The effect of ADP on endogenous respiration by minced red muscle of goldfish. Minced red muscle was incubated at 20°C in standard incubation medium (pH 7.4). Oxygen uptake was measured polarographically for 5 min. Each point represents the mean of at least duplicate measurements, using red muscle of one goldfish from group I.

the endogenous respiration by added glucose 6-P. The inhibition of cellular respiration as a reaction to glucose addition is often called the Crabtree, or reversed-Pasteur effect. It is primarily known from Ehrlich-ascites tumour cells (Crabtree, 1929). In non-neoplastic cells it is less frequently reported in a wide variety of tissues (Ibsen, 1961). Some characteristics of the glucose 6-P induced inhibition of respiration were investigated. Suppression of oxygen uptake could be released by ADP and, to a minor extent, by succinate. Glutamate had no effect. The activation by ADP is concentration dependent with an optimum at 0.5 mM ADP (Fig. 1).

Total CO_2 production

Aerobic and anaerobic production rates of metabolic CO_2 by minced red muscle were determined manometrically by means of a pressure transducer. Quantitative results are given in Table 2. In contrast to the rate of oxygen uptake, the total CO_2 production is stimulated after addition of glucose 6-P. The opposite effects of glucose 6-P on the rates of oxygen uptake and CO_2 production cause a high respiratory quotient: 1.60 for the endogenous, and 2.26 for the glucose 6-P induced respiration (Table 2).

Replenishing the medium with glutamate or ADP, the respiratory quotient becomes 1.21 and 1.04 respectively (Table 3). Anaerobic production of metabolic CO_2 falls to about 30% of the aerobic rate unless glutamate is added (Table 3). Addition of glutamate enhances anaerobic CO_2 production by minced red muscle to 77% of the aerobic rate.

Oxidation of [U-^{14}C] labelled substrates

Rates of $^{14}CO_2$ production from 2.5 mM [U-^{14}C] glucose 6-P and 1 mM [U-^{14}C] glutamate by minced red muscle of three groups of goldfish are presented in Table 4. The rates of $^{14}CO_2$ production by minced red muscle demonstrate similar group dependent vari-

Table 2. The rates of total CO_2 production and oxygen uptake by minced red muscle of goldfish

Substrates (final concn)	Oxygen uptake (nmol O_2/min/100 mg wet wt)	RQ	Total CO_2 production (nmol CO_2/min/100 mg wet wt) Aerobic	Anaerobic
Endogenous	42.1 ± 6.6	1.60	67.6 ± 0.1	25.9 ± 2.7
2.5 mM glucose 6-P	35.3 ± 3.2	2.26	79.8 ± 1.8	28.3 ± 5.1

Minced red muscle was incubated at 20°C in standard incubation medium (pH 7.4) for 15–60 min. Oxygen uptake was measured polarographically. Total CO_2 production was measured manometrically. Each value is the mean of duplicate incubations, using minced red muscle of 1 goldfish. Results are expressed as the mean ± SD of two independent experiments.

Table 3. The ratio between anaerobic and aerobic CO_2 production and the respiratory quotient of goldfish red muscle

Substrates (final concn)	Uniform ^{14}C label	Ratio of anaerobic and aerobic CO_2 production $^{14}CO_2$	$t\,CO_2$	Respiratory quotient RQ*
Endogenous	—	—	0.34 ± 0.07 (4)	1.42 ± 0.33 (4)
2.5 mM glucose 6-P	G 6-P	0.71 ± 0.07 (3)	0.30 ± 0.10 (3)	2.67 ± 0.71 (3)
2.5 mM glucose 6-P + 1 mM glutamate	G 6-P Glut	0.78 ± 0.20 (6) 0.57 ± 0.14 (5)	0.77 ± 0.12 (6)	1.21 ± 0.10 (6)
2.5 mM glucose 6-P + 1 mM ADP	G 6-P	0.92 ± 0.23 (5)	0.37 ± 0.01 (2)	1.04 ± 0.02 (2)

Minced red muscle of goldfish was incubated at 20°C in standard incubation medium (pH 7.4) for 60–120 min. Substrates were added as indicated; for determination of $^{14}CO_2$ production 0.05 μCi of [U-^{14}C] labelled substrates was added. Oxygen uptake was measured polarographically. Total (t) CO_2 production was measured manometrically. Results are expressed as the mean ± SD of n independent experiments.

Table 4. The rates of aerobic and anaerobic $^{14}CO_2$ production from [U-^{14}C] glucose 6-P and [U-^{14}C] glutamate by red muscle tissue of goldfish and variations between groups of fish

Group*	Number of fish (n)	Uniform ^{14}C label	Specific activity† Aerobic	Specific activity† Anaerobic	Ratio‡ Anaerobic/aerobic $^{14}CO_2$
I	3	G 6-P	1.95±0.51	1.49±0.31§	0.81±0.29
	3	Glut	1.54±0.94	0.85±0.53§	0.56±0.20
II	10	G 6-P	3.14±1.04	2.23±0.97‖	0.70±0.15
III	5	G 6-P	2.06±0.54	1.77±0.50‖	0.87±0.20

*Groups as indicated in Table 1.
†nmol substrate converted to $^{14}CO_2$/min/100 mg wet wt.
‡Ratio expressed as mean ±SD of individuals ratios.
§Activities obtained from duplicate incubations at various times (30–180 min).
‖Activities obtained from multiplicate incubations at 120 min.
Minced red muscle was incubated at 20°C in standard incubation medium (pH 7.4) supplemented with 2.5 mM glucose 6-P, 1 mM glutamate and 0.05 μCi ^{14}C labelled substrate. Each value was the mean of several incubations using minced red muscle of 1 goldfish per experiment. Results are expressed as the mean ±SD of n independent experiments.

ations as were noted earlier on the respiration rates. The ratio between anaerobic and aerobic $^{14}CO_2$ production is much less variable between the groups of fish. Effects of anoxia on the release of $^{14}CO_2$ from [U-^{14}C] glucose 6-P and [U-^{14}C] glutamate by minced red muscle of goldfish are shown in Tables 3 and 4. In Table 3 the effects of glutamate and ADP on the ratio between anaerobic and aerobic $^{14}CO_2$ from [U-^{14}C] glucose 6-P are presented. Glutamate has almost no effect on the $^{14}CO_2$ production ratio from [U-^{14}C] glucose 6-P. Combining ADP with [U-^{14}C] glucose 6-P the ratio becomes 0.92 (Table 3), thus almost eliminating the effect of anoxia. Under aerobic conditions glucose 6-P and glutamate are oxidized to CO_2 at almost equal rate (Table 4). Anoxia suppresses $^{14}CO_2$ production from [U-^{14}C] glucose 6-P to 81% and from [U-^{14}C] glutamate to 56% of the aerobic rate (Table 4, group I).

Metabolic pathways in the CO_2 production from glucose 6-P

According to the method described by Katz and Wood (1963) the activity of the hexose monophosphate shunt in red muscle of goldfish was estimated by determination of the $^{14}CO_2$ production ratio from [6-^{14}C] and [1-^{14}C] glucose 6-P. Under aerobic and anaerobic conditions the $^{14}CO_2$ production ratio from C6/C1 is approximately one (Table 5). The ratio between anaerobic and aerobic $^{14}CO_2$, produced from [1-^{14}C] or [6-^{14}C] glucose 6-P is almost equal: 1.36 and 1.49 respectively (Table 5), but considerably higher than the $^{14}CO_2$ production ratio from [U-^{14}C] glucose 6-P of 0.81 (Table 4).

The participation of the Emden–Meyerhof pathway to aerobic and anaerobic glucose metabolism in goldfish red muscle was roughly estimated by iodoacetic acid (IAA) poisoning of glyceraldehyde 3-P dehydrogenase. As shown in Fig. 2 the oxidation of [U-^{14}C] glucose 6-P is completely inhibited by low concentrations of IAA.

Table 5. The rates of aerobic and anaerobic $^{14}CO_2$ production from [1-^{14}C], [6-^{14}C] glucose 6-P by red muscle tissue of goldfish

Condition	Glucose 6-P label	Specific activity*	$^{14}CO_2$ from C-6 / $^{14}CO_2$ from C-1
Aerobic	1-^{14}C	2.36±0.44	1.02
	6-^{14}C	2.40±0.16	
Anaerobic	1-^{14}C	3.16±0.31	1.13
	6-^{14}C	3.58±0.43	

*nmol substrate converted to $^{14}CO_2$/min/100 mg wet wt.
Minced red muscle was incubated at 20°C in standard incubation medium (pH 7.4) for 30–90 min. The medium was supplemented with 5 mM glucose 6-P and 0.025 μCi of [1-^{14}C] glucose 6-P or 0.05 μCi of [6-^{14}C] glucose 6-P. Results are expressed as the mean ±SD of 4 incubations

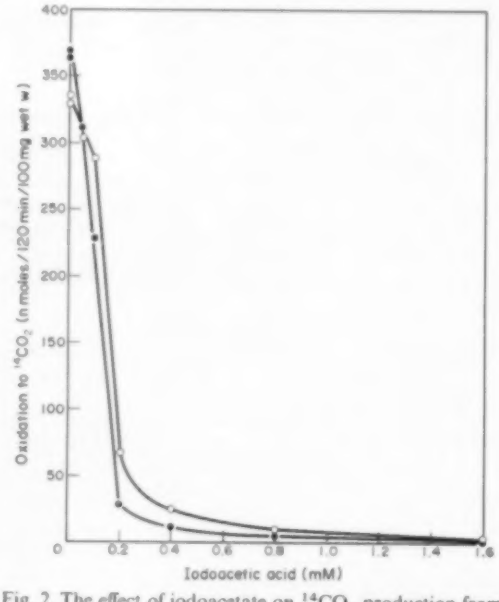

Fig. 2. The effect of iodoacetate on $^{14}CO_2$ production from [U-^{14}C] glucose 6-P by minced red muscle of goldfish. Minced red muscle (of group III fish) was incubated at 20°C for 120 min in standard incubation medium (pH 7.4) supplemented with 2.5 mM [U-^{14}C] glucose 6-P (0.05 μCi), 1 mM glutamate and various concentrations of iodacetate. (●), aerobic incubation; (○), anaerobic incubation.

DISCUSSION

Oxidative properties of minced red muscle

Data were acquired using minced red muscle, according to Hess and Brandt (1970). In contrast to red muscle slices, this preparation was found to demonstrate a high degree of homogeneity and an active oxidative metabolism. Incubated in a medium of intracellular Na^+/K^+ composition, minced muscle may be considered as a suspension of intact intracellular particles, including mitochondria, in diluted cytoplasm. Mitochondrial intactness was demonstrated by succinate and ADP induced activation of oxygen uptake (Fig. 1). Significant variations in the rates of oxygen uptake and $^{14}CO_2$ production by minced red muscle were found between several groups of goldfish. These variations were of only quantitative importance (Tables 1 and 4). The variability may be due to season dependent alterations of metabolic activities. The existence of the latter is known from the seasonal variation of oxygen uptake by goldfish gills (Ekberg, 1956), which is not due to temperature effects.

Crabtree effect and respiratory quotient

The rate of oxygen uptake by goldfish red muscle tissue *in vitro* was suppressed to 75% of the endogenous rate after addition of glucose 6-P (Table 1). The Crabtree effect in goldfish red muscle and in non-neoplastic tissues of mammals is of the same order of magnitude (Ibsen, 1961).

The mechanism of the glycolytically produced inhibition of respiration is not yet fully understood but it is generally accepted that ADP or phosphate becomes limiting at the sites of oxidative phosphorylation (Ibsen, 1961). In tumour cells the decrease of oxygen uptake by glucose addition is associated with an excessive high rate of glycolysis. The Crabtree effect is thought to be due to an indirect competition of glycolysis and oxidative phosphorylation, involving the availability of common intermediates in the cytoplasmic and the mitochondrial compartment.

In contrast to the rate of oxygen uptake, total CO_2 production is stimulated by glucose 6-P addition (Table 2). As a result of the opposite effects of glucose 6-P on oxygen uptake and CO_2 production, the respiratory quotient becomes significantly greater than one (Table 3). This was also observed with the intestine of Ascaris (Harpur and Jackson, 1976). In Erhlich ascites tumour cells both oxygen uptake and CO_2 production are inhibited by glucose addition (Crabtree, 1929), suggesting a different mechanism of metabolic CO_2 production. The increase of the RQ to values greater than one points to an incomplete oxidation of glucose 6-P. In goldfish red muscle this may be associated to anaerobic decarboxylation activity of the mitochondria, which are able to convert pyruvate into acetaldehyde (Mourik *et al.*, 1982). Diffusing into the cytoplasm, acetaldehyde may serve as an electron acceptor during anoxia. Ethanol and CO_2 are excreted. When the cellular oxygen tension becomes limiting, this CO_2-producing system possibly supports the maintenance of the redox balance, thus explaining the high RQ values of swimming goldfish at very low pO_2, as observed by Kutty (1968).

Incomplete oxidation of glucose 6-P by minced red muscle of goldfish is overcome by the addition of ADP or glutamate. The stimulation of aerobic mitochondrial activities is expressed by RQ values of 1.04 and 1.21 respectively (Table 3).

Anaerobic CO_2 production

As in the whole animal, anaerobic metabolism of goldfish red muscle comes to expression by the release of metabolic CO_2 (Tables 2 and 4).

In the presence of endogenous substrates, glucose 6-P or glucose 6-P plus ADP, the ratio between anaerobic and aerobic decarboxylation activity by minced red muscle is 0.30–0.37 (Table 3). Combining glucose 6-P and glutamate, the ratio becomes 0.77, suggesting the capability of goldfish red muscle to utilize both carbohydrate and amino acid as a carbon source during anoxia. The separate contribution of glucose 6-P and glutamate metabolism to the production of CO_2 was investigated with [U-^{14}C] labelled substrates. As shown in Table 4 the $^{14}CO_2$ production rates from glucose 6-P and glutamate in group I are of the same order of magnitude. These rates are comparable to the rate of [1-^{14}C] lactate decarboxylation by red muscle slices of rainbow trout: 1.71 nmol substrate conversion to $^{14}CO_2$/min/100 mg wet wt at 15°C (Bilinski and Jonas, 1972).

Comparing the ratio between anaerobic and aerobic $^{14}CO_2$ production by minced red muscle of goldfish in the presence of 2.5 mM glucose 6-P and 1 mM glutamate it may be concluded that the oxidation of [U-^{14}C] glucose 6-P is less affected by anoxia than the oxidation of [U-^{14}C] glutamate with a ratio of 0.81 and 0.56 respectively (Table 4, group I). Combining [U-^{14}C] glucose 6-P and ADP, the ratio between anaerobic and aerobic $^{14}CO_2$ production is 0.92, thus overcoming the inhibitory effect of anoxia (Table 3). A similar stimulation of anaerobic metabolism by ADP was also observed on the rates of pyruvate uptake and acetaldehyde formation by goldfish red muscle mitochondria (Mourik *et al.*, 1982). So it appears that ADP is a positive modulator of anaerobic glucose metabolism in goldfish red muscle.

Enzymatic pathways in glucose catabolism

Aerobic and anaerobic glucose oxidation were examined in more detail to estimate the involvement of the hexose monophosphate shunt and the Emden–Meyerhof pathway. No activity of the hexose monophosphate shunt in goldfish muscle could be determined. This was deduced from the ratio of $^{14}CO_2$, produced from [6-^{14}C] and [1-^{14}C] glucose 6-P, of approximately one (Table 5). For aerobic metabolism this agrees with data obtained by Hochachka (1961) and Brown (1960) on whole goldfish and carp respectively. Activity of the hexose monophosphate shunt in anaerobic metabolism was observed in larvae of the insect *Callitroga macellaria* (Meyer, 1977). During exposure of *Callitroga* larvae to anoxia, the C6/C1 ratio in $^{14}CO_2$ production decreased to 0.01 as compared to 0.98 in the control. In goldfish red muscle no contribution of the shunt to anaerobic $^{14}CO_2$ production was demonstrated (Table 5). The classical sequence of the glycolytic pathway in goldfish red muscle was roughly estimated by iodoacetate inhibition of glyceraldehyde 3-P dehydrogenase. Both aerobic and anaerobic $^{14}CO_2$ production from glucose 6-P were completely inhibited by low concen-

trations of iodoacetate, suggesting an equal dependency on the glycolytic dehydrogenation reaction.

CONCLUSION

It may be concluded that red skeletal muscle of goldfish is capable of releasing CO_2 from glucose 6-P and glutamate under aerobic and anaerobic conditions. The rate of CO_2 production from [U-^{14}C] glucose 6-P is less affected by anoxia than the CO_2 production from [U-^{14}C] glutamate suggesting a preferential but not exclusive catabolism of carbohydrate by the anoxic red muscle. This is in accordance with observations *in vivo*. Glycogen stores are markedly diminished (Van den Thillart *et al.*, 1980) and $^{14}CO_2$ release from [U-^{14}C] glucose is stimulated 3–4-fold by anoxia (Van den Thillart, 1977). On the other hand, protein and amino acid catabolism as catalysed by the mitochondria appears to be not completely blocked. In the anoxic goldfish this is demonstrated by the continuation of the NH_3-production rate (Van den Thillart, 1977). Anaerobic functioning of mitochondria in the goldfish may be not a unique phenomenon among vertebrates. The beating of anaerobic rat heart is elevated when, besides glucose as an essential factor, select TCA-cycle intermediates are added to the perfusate (Cascarano *et al.*, 1968). The improvement of anaerobic beating is caused by a stimulation of the mitochondrial ATP production (Penney and Cascarano, 1970). In the ventricular papillary muscle of rabbit heart anaerobic synthesis of succinate from aspartate and glutamate was demonstrated (Taegtmeyer, 1978), although the significance in mammalian tissue is still controversial. In goldfish red muscle, however, the role of mitochondria appears to be of considerable importance in anaerobic energy metabolism.

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CELL MEDIATED IMMUNE RESPONSE OF *BUFO ARENARUM* AND *LEPTODACTYLUS OCELLATUS* (AMPHIBIA, ANURA) TO MYCOBACTERIAL ANTIGENS

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Abstract—1. The lymphocyte transformation test was applied to the study of cell mediated immune (CMI) response *in vitro* to mycobacterial antigens in *Bufo arenarum* and *Leptodactylus ocellatus* previously inoculated with a suspension of *Mycobacterium tuberculosis*.

2. The stimulated samples of blood both in *B. arenarum* and *L. ocellatus* showed a significant increase in the percent of blastic dedifferentiation (almost 100% with respect to controls) suggesting the presence of a positive CMI response to PPD.

3. The differences were observed on the fifth day of incubation and lasted until the seventh day.

INTRODUCTION

Adult amphibians constitute an important link in the study of the phylogeny of the immune system of vertebrates, especially when considered in the context of the transition from aquatic to terrestrial habits. They themselves are also of special interest because of their free-living embryonic and larval stages which make it feasible to obtain additional information relating to the ontogeny of their immune response (Cohen, 1976; Katagiri, 1978; Tam *et al.*, 1976).

Both antibody (Atwell and Marchalonis, 1976; Cooper, 1976) and cell mediated immune responses (CMI) (Hadji-Azimi, 1979) have been described in Amphibia. The research on CMI in amphibians have been oriented toward the study of skin allograft rejection. There are results on rejection phenomena of chronic evolution in Apoda and Urodela (Horton, 1971) and of acute rejection response to allogeneic grafts in several species of Anura (Cohen, 1971).

Because of the similarity of their development pattern and the degree of thymus maturation, the existence of an equivalence between the CMI mechanisms of amphibians and those of mammals is accepted; this similarity was remarkable when it was referred to tissue grafts in particular.

The *in vitro* lymphocitary transformation test (LTT) is considered as an acceptable criterion in the evaluation of the CMI in mammals (Warr and Marchalonis, 1978). Several authors have applied the LTT to Amphibia, finding 30–70% of lymphoblastic dedifferentiation when facing nonspecific mitogens such as phytohaemagglutinin or Concanavalin A (Donnelly and Cohen, 1979; Horton *et al.*, 1980; Wright *et al.*, 1979; Wright and Cooper, 1979).

The present study was carried out to determine the CMI to mycobacterial antigens in two South American batrachians, evaluating it in *in vitro* conditions by the LTT in animals previously immunized with that antigen.

MATERIALS AND METHODS

Animals came from Buenos Aires and Santa Fe Provinces, Argentina. Their body mass (mean ± SEM) was 67.4 ± 9.5 g ($N = 9$) for toads and 84.8 ± 10.5 g ($N = 8$) for frogs. In order to avoid the seasonal variations of the hematopoiesis (Varela and Mancini, 1938; Varela and Sellarés, 1937, 1938), all the experiments were carried out in winter, irrespective of the sex of the animals.

Each animal was subcutaneously inoculated with a suspension (1.5 mg/ml) of *Mycobacterium tuberculosis* (BCG Doerr, Dupomar Laboratories) containing Freund's incomplete adjuvant (Difco Laboratories) diluted 1:1, at a dose of 0.01 ml/*g* body wt. Seven days later, blood was aseptically obtained by cardiac puncture with heparinized plastic syringes, and allowed to sediment for 30 min in test tubes placed in a culture chamber at room temperature (15–17°C).

The supernatants were transferred to culture tubes in the proportion of 0.5 ml of plasma rich in cells, 0.45 ml of TC-199 culture medium (Difco Laboratories) and 0.05 ml of fetal calf serum (Laboratorio Immunochemia, Buenos Aires). The stimulated cultures received as antigen 0.05 ml (5 IU) of a purified proteic preparation (PPD) from *M. tuberculosis* var. *hominis* (0.02 μg of PPD = 1 IU), prepared in the Centro Panamericano de Zoonosis (CEPANZO), Buenos Aires.

The cultures were incubated at room temperature for 120 hr and at the end of that time a sample was removed. The procedure was repeated 48 hr later. An aliquot of each of these samples (0.3 ml) was mixed with 0.5 ml of a solution prepared mixing one part of isotonic saline and two parts of distilled water. The mixtures were allowed to rest for 30 min and then were centrifuged for 5 min at 50 *g*.

The supernatants were discarded and the remaining pellet was mixed with 0.5 ml of Carnoy's solution (methanol–glacial acetic acid 5:1). The culture was left undisturbed for 30 min and centrifuged again (5 min at 50 *g*). The supernatants were discarded and the whole procedure was repeated once more. All cultures were prepared in duplicate.

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Table 1. Blastic dedifferentiation in adult *Bufo arenarum*

	5th day	7th day	Differences*
Controls	6.8 ± 0.6	5.8 ± 0.6	−1.0 ± 0.2†
Stimulated	11.2 ± 0.8	9.3 ± 0.9	−1.9 ± 0.5‡
Differences*	+4.4 ± 0.8§	+3.5 ± 0.6‖	

Data in mean percent ± SEM ($N = 9$).
*Mean difference of paired data ± SEM.
†$P < 0.005$; ‡$P = 0.005$; §$P < 0.001$; ‖$P < 0.001$.

From the final suspension a smear on cold glass was made, also in duplicate. The preparations were left to dry and then stained with May–Grünwald–Giemsa stain for microscopic observation. The percentage of lymphoblasts in relation to the total number of counted lymphocytes was calculated from the mean of duplicates; a minimum of 500 cells was examined for each preparation.

The statistical analysis of the results was carried out by means of Student's *t* test (Snedecor and Cochran, 1969).

RESULTS AND DISCUSSION

Results are presented in Tables 1 and 2. In the toad *Bufo arenarum* there was a highly significant difference between the percentage of spontaneous blastic dedifferentiation and that observed in samples stimulated with PPD, both on the fifth and seventh days. Similar findings were observed in the frog *Leptodactylus ocellatus*.

In comparing the differences between the controls and the stimulated samples on the fifth and seventh days, it became evident that the percentages of stimulation were nearly identical in both species.

The responses found both in controls and in stimulated samples of *L. ocellatus* on the fifth day remained unmodified throughout the seventh day. On the contrary, the behaviour of *B. arenarum* was different since on the seventh day the differences in both experimental groups were significantly reduced. These dissimilarities might be accounted for as a species-dependent response.

Our data clearly show the capacity of peripheral blood lymphocytes of *B. arenarum* and *L. ocellatus* to respond *in vitro* to antigens of *Mycobacterium tuberculosis* (PPD); a similar response to PPD has been described for tymocytes of *Xenopus* (Donnelly and Cohen, 1979; Horton *et al.*, 1980). Nevertheless it is noteworthy to call attention to the fact that the response to PPD described herein has been considerably lower than that described for other amphibians' blood to nonspecific mitogens (Donnelly and Cohen, 1979).

It is also worthwhile remarking that the timing of the response of *B. arenarum* and *L. ocellatus* was quite similar to that observed in mammals. With regard to these findings Wright and Cooper (1979) have concluded that ". . . vertebrate lymphocytes have not undergone significant phylogenetic changes since the first tetrapods evolved approximately 350 million years ago".

Table 2. Blastic dedifferentiation in adult *Leptodactylus ocellatus*.

	5th day	7th day	Differences*
Controls	5.8 ± 0.5	5.5 ± 0.5	−0.3 ± 0.4†
Stimulated	10.3 ± 1.1	9.3 ± 0.5	−1.0 ± 0.9†
Differences*	+4.5 ± 0.7‡	+3.8 ± 0.6§	

Data in mean percent ± SEM ($N = 8$).
*Mean difference of paired data ± SEM.
†Statistically nonsignificant; ‡$P < 0.001$; §$P < 0.001$.

The existence of a positive response to PPD after seven days of immunization with BCG could be considered as an evidence of previous contact of the animals with mycobacterial antigens. It is interesting to mention, for instance, that the presence of saprophytic and pathogenic mycobacteria on the skin of amphibians has already been reported (Glorioso *et al.*, 1974; Schwabacher, 1959).

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EXTERNAL FACTORS CONTROLLING ANNUAL TESTOSTERONE AND THYROXINE CYCLES IN THE EDIBLE DORMOUSE *GLIS GLIS*

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(*Received* 6 *April* 1983)

Abstract—1. Annual patterns of hibernation, body weight, plasma testosterone and thyroxine were measured in two groups of edible dormice exposed to a constant photoperiod and to either environmental temperature (I) or constant cold (II).

2. In group I, the four functions displayed annual cycles and the two hormonal cycles were parallel and restricted to the aroused state, while in group II only irregular infradian fluctuations were observed.

3. However, a body temperature of 30°C remained the critical lower limit enabling the start of the testosterone cycles for the two groups.

4. Respective roles of endogenous and synchronizing mechanisms in controlling biological rhythms are discussed.

INTRODUCTION

Hibernators have long been explored as models particularly suited to the study of the adaptive metabolic and behavioral strategies developed by mammals when facing extreme environmental conditions (Kayser, 1970). More recently, interest has increasingly focussed on the hormonal cycles that may be connected with or control the hibernating cycles. From available data, it is clear that annual endocrine cycles in hibernators do not exhibit a single general pattern, since these cycles display a wide range of endocrine correlations. Only extensive comparison of the endocrine secretion levels in various hibernating species can therefore elucidate the mechanisms by which external and internal factors intervene in the control of the processes involved in adapting to extreme environmental conditions.

In an initial attempt to study the endocrine correlates of hibernation in the edible dormouse, we explored the annual profiles of plasma concentrations of testosterone and thyroxine in dormice kept in a natural environment (Jallageas and Assenmacher, 1983). Two salient characteristics emerged from the study: 1. testosterone and thyroxine levels displayed clear annual cycles running parallel both to each other and to the ambient photoperiod and temperature, and 2. unlike several other hibernators, the annual rise in the levels of both hormones in the edible dormouse never preceded the terminal arousal from hibernation. Furthermore, 30°C was clearly the critical minimal body temperature required for this annual arousal.

The present series of experiments was designed to clarify further the respective role of the annual photoperiod and temperature cycles in the control of the endocrine cycles.

MATERIALS AND METHODS

Twenty-eight adult male edible dormice were captured during the late summer of 1980 in the Forest of Chizé in Western France at the Centre d'Etudes Biologiques des Animaux Sauvages belonging to the Centre National de la Recherche Scientifique. After transfer to our Laboratory, they were divided into two experimental groups in December 1980. The first group comprising 16 animals was exposed to environmental outdoor temperatures in a windowless thin-walled experimental chamber equipped with an automatic device ensuring a constant photoperiod of 12 hr light (100 lx at 0700 hr) and 12 hr darkness. Figure 1 gives the annual variations in the indoor ambient temperature (*AT*), which ranged from 4.5°C in December to 28°C in June, July and August during the period covered by the experiment (December 1980 to February 1982). These indoor temperatures were fairly similar to the outdoor temperatures. A second group of 12 dormice was kept in a room whose temperature was maintained at 5.8 ± 0.3°C, and exposed to the same 12 hr light–12 hr dark photoperiod as group I. All the animals were housed singly in wire-mesh cages (65 × 34 × 30 cm), each provided with a nest-box

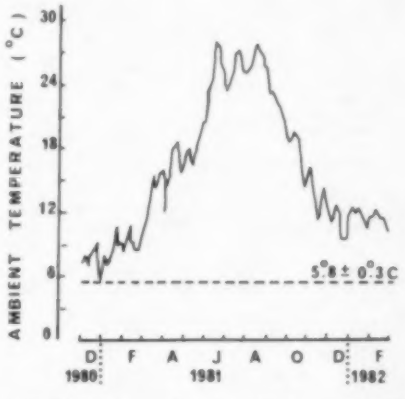

Fig. 1. Ambient temperature in animal rooms. Solid lines: group I, housed in a thin-walled room directly exposed to outdoor temperatures. Broken lines: group II housed in a cold room.

furnished with straw, and with an automatic device for drinking water. Animals had free access to a standard hamster chow (U.A.R., Provimi, Paris), and were given a few apples twice a week. Throughout the study, their individual wake/dormancy behaviour was checked daily. In addition, on a fixed date each month between 0930 hr and 1000 hr, each animal was weighed, its rectal temperature was quickly recorded with a Digi-sense thermometer as used by Jallageas and Assenmacher (1983), and 1 ml blood samples were taken by heart puncture, and centrifuged. These three procedures were completed in less than 2 min. In alert animals they were performed under fluothane (I.C.I. Pharmaceuticals) anesthesia, whereas dormant animals were handled directly (see Young *et al.*, 1979). Plasma vials were stored at −30°C until use.

Plasma testosterone levels were measured in duplicate on individual samples of 150–450 μl depending on the concentration, with RIA kits from the Commissariat à l'Energie Atomique, Saclay, France. The only significant cross-reaction for this antibody concerned dihydrotestosterone (75% at half binding levels). The lowest detectable testosterone concentration was 5 pg/tube and the intra- and inter-assay coefficients of variation were 5.6% and 6.5% respectively.

For plasma thyroxine assays, we used competitive protein-binding (Astier *et al.*, 1978), with pregnant human thyroxine binding-globulin as binding protein, and L-thyroxine (Calbiochem, USA) as standard hormone. Each assay was conducted on 250 μl of plasma. The lowest detectable amount of thyroxine was 0.4 ng/tube and the intra- and inter-assay coefficients of variation were 5.1% and 5.6% respectively.

Results are expressed as means ±SEM. For statistical comparisons, we used either parametric tests (Student's test for two means, or one-way ANOVA followed by a Fisher test for more than two groups), or non parametric tests (Wilcoxon's W test or the Krukskall–Wallis test) depending on data distribution. To evaluate specific correlations, we referred to Spearman's rank correlations.

RESULTS

Group I: 12 hr light–12 hr dark and natural environmental temperatures

1. *Hibernation* (Fig. 2b). The dormice in this group displayed a clear annual hibernation cycle (Fig. 2b) that closely followed the pattern of the specimens studied in previous experiments conducted in the open air (Jallageas and Assenmacher, 1983) on animals which served as controls for the present series (Fig. 2a). From early June until early September, all the animals in group I were alert. A few entered dormancy during September and the number of lethargic specimens increased during October. By November or December, all dormice were hibernating. Throughout the hibernation period but with increasing frequency as the vernal arousal approached, intermediate stages of torpor alternated with periodic bouts of arousal. In January, a few animals emerged from hibernation, but throughout February and March, group I still comprised twice as many lethargic as aroused dormice.

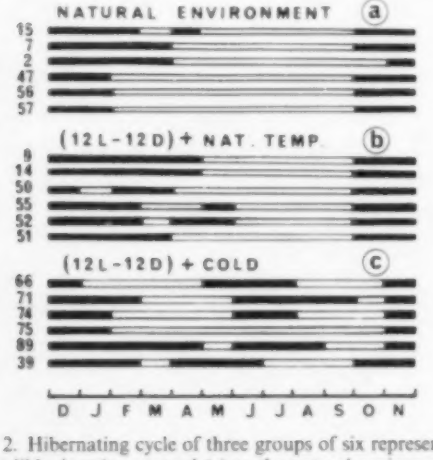

Fig. 2. Hibernating cycle of three groups of six representative edible dormice exposed (a) to the natural environment (from Jallageas and Assenmacher, 1983), (b) to a constant photoperiod and varying seasonal temperatures (group I. Fig. 1) and (c) to a constant photoperiod and cold environment (group II).

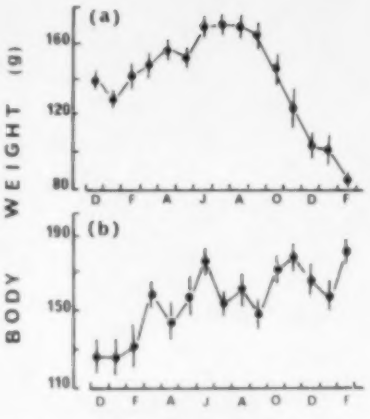

Fig. 3. Body weight cycle of edible dormice exposed to (a) constant photoperiod and varying seasonal temperatures (group I) or (b) to constant photoperiod and cold (group II).

2. *Body weight* (Fig. 3a). Compared to the general profile of body weight variations in hibernating mammals, the annual body weight cycle of the dormice in group I was characterized by marked fattening which culminated in the preparatory phase of hibernation in September and then decreased sharply during the phase of entry into dormancy. Intriguingly, however, this body weight cycle displayed an essentially monophasic profile, with only a transient plateau in May and June, thus contrasting with the biphasic cycle of the earlier series of animals housed in the open air (Jallageas and Assenmacher, 1983), whose body weight displayed a temporary phase of decline in June and July, interposed between the two ascending phases which respectively started at arousal and during prehibernation.

3. *Plasma testosterone* (Fig. 4). In group I animals plasma testosterone displayed a clear annual cycle very similar to that for the animals housed in the open. High testosterone levels were only observed in group I from March to the end of July, with a maximum in May. However, Fig. 4a clearly shows that the vernal increase in plasma testosterone was restricted to aroused animals, since it was never seen in hypothermic dormice. Figure 4a further indicates that the sharp decrease in circulating androgen levels preceded the onset of hibernation by a few months, as concentrations near to the baseline were already

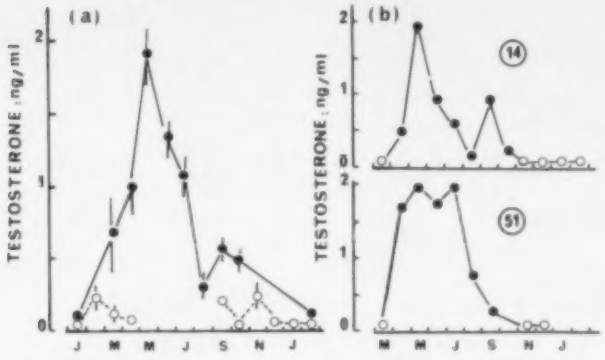

Fig. 4. Annual cycle of plasma testosterone concentrations in edible dormice exposed to a constant photoperiod and varying seasonal temperatures (group I, housed as stated in Fig. 1). Black circles: aroused animals; open circles: dormant or sleepy dormice with body temperatures < 30°C. (a) Means ± SEM for aroused and lethargic animals respectively, (b) two representative profiles: animals 14 and 51.

recorded in August, i.e. in fully alert animals. A temporary moderate resurgence of plasma testosterone was observed in October and November in some of the animals which had stayed aroused in early autumn but never in those which had already entered dormancy at this period. Representative specimens of those two different patterns are illustrated in Fig. 4b.

4. *Plasma thyroxine* (Fig. 5). Concentrations of plasma thyroxine in group I animals also displayed an annual cycle reminiscent of that of the preceding series of animals housed in the open (Jallageas and Assenmacher, 1983). Accordingly, plasma thyroxine remained at its maximum from April to the end of July, i.e. at about twice the baseline level (Fig. 5a). This high plateau for plasma thyroxine corresponded to the period during which most dormice were fully awake and it was interesting to note that the sharp drop in thyroxine levels in August coincided with the decline of plasma testosterone. Statistical estimations of the rank correlations (Spearman) for both hormones in individual animals showed a fairly close correlation between them ($P < 0.001$; $\rho = 0.5$; $N = 79$). Like plasma testosterone, plasma thyroxine displayed a transient resurgence in early autumn (Fig. 5a). Curiously, however, this autumnal thyroxine peak—unlike that for testosterone (cf. Fig. 4b)—appeared a constant feature, as illustrated for two representative animals in Fig. 5b. Consequently this pattern was not always connected with the plasma testosterone level, nor with the wake/torpor cycles (Fig. 5b).

Group II: 12 hr light–12 hr dark and a constant cold environment (5.8°C)

When a low ambient temperature was maintained and (as for group I) the photoperiod did not vary, the pattern of the physiological parameters measured altered dramatically.

1. *Hibernation* (Fig. 2c). Although all group II animals displayed alternative phases of dormancy and activity, these alternating sequences were essentially polyphasic and were not related to each other or to the season. Close examination of the data showed that in December, January and September, there were at least 30% more lethargic than aroused specimens, whereas the reverse ratio was observed from February to May, with equal proportions of lethargic and alert animals in June. In addition, the total period of dormancy in group II for the 15 months of the experiment was significantly longer than that of group I and also of the earlier series of animals kept outdoors (Jallageas and Assenmacher, 1983).

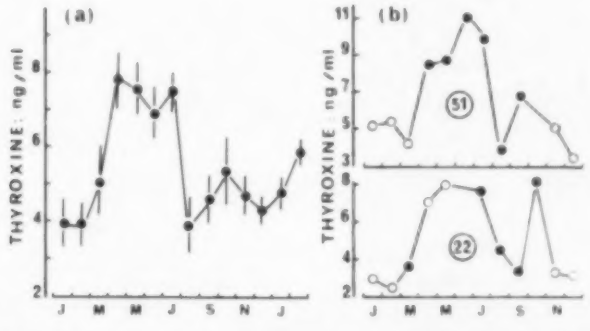

Fig. 5. Annual cycle of the plasma thyroxine concentration in edible dormice exposed to a constant photoperiod and varying seasonal temperatures. Symbols as in Fig. 4.

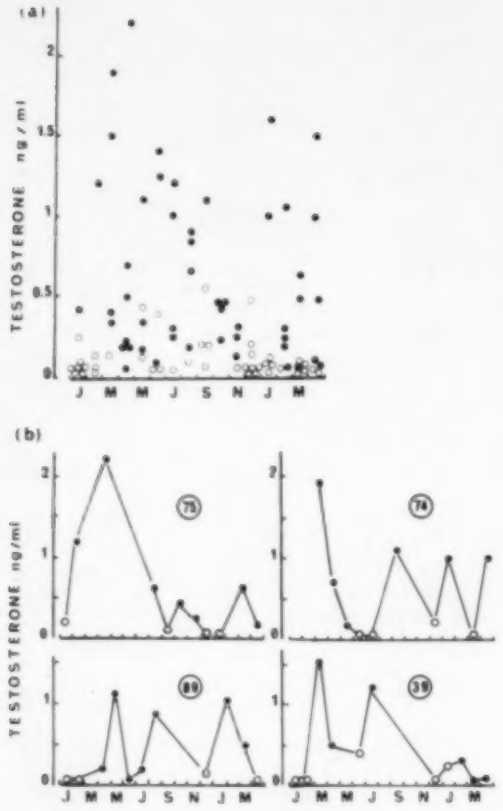

Fig. 6. Plasma testosterone concentrations in edible dormice exposed to an unvarying photoperiod (12 hr light–12 hr dark) and constant cold (5.8 ± 0.3°C). Black circles: aroused animals; open circles: dormant or sleepy dormice with body temperature < 30°C. (a) High and low individual concentrations were measured throughout the year, (b) four representative individual profiles: animals 75, 74, 89 and 39.

2. *Body weight* (Fig. 3b). As in group I, the body weight of group II animals displayed definite fluctuations. However, their pattern was very irregular, and neither annual nor biannual cyclicity emerged from this background of brief fluctuations over periods of 2–4 months. Taken together, body weight data also showed a trend towards increased fattening, since, from March 1981, most values exceeded 150 g, a level never observed in animals undergoing prolonged hibernation.

3. *Plasma testosterone* (Fig. 6). No annual cycle was apparent for plasma testosterone concentrations in group II. Figure 6a shows that, as in group I and in the earlier outdoor control series (Jallageas and Assenmacher, 1983), lethargic animals were always characterized by low testosterone levels ($\leqslant 0.5$ ng/ml). However, since, unlike group I and the earlier series, group II animals exhibited hibernation sequences throughout most of the year, they also displayed very low testosterone levels at all seasons. Conversely, in aroused dormice, increased testosterone levels were measured throughout the year except during October and November. More enlightening are the data from individual group II animals, as they clearly show the occurrence of short irregular fluctuations in plasma testosterone levels (Fig. 6b). Three features were common to all animals: 1. periods of dormancy, and more precisely body temperatures below 30°C, which always occurred in conjunction with low plasma testosterone levels; 2. phases of increased levels of testosterone, always concomitant with arousal phases; and 3. declining testosterone values that always preceded entry into lethargy.

4. *Plasma thyroxine* (Fig. 7). Like the testosterone titers, plasma thyroxine levels in group II did not display one annual cycle (Fig. 7a), but three to four irregular cycles (Fig. 7b). At first sight these fluctuations were evocative of the short cycles described above for plasma testosterone. However,

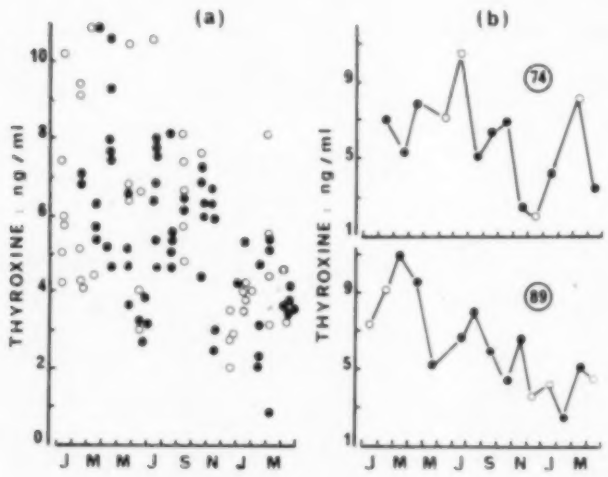

Fig. 7. Plasma thyroxine concentrations in edible dormice exposed to a constant photoperiod and cold. Symbols as in Fig. 6.

closer examination of individual data clearly revealed that there was no correlation between either thyroxine and testosterone fluctuations (Spearman's $\rho = 0.25$; $P > 0.05$) or plasma thyroxine levels and the phases of the wake/torpor cycle. This absence of correlation was strikingly evident in the July measurements for animal 74 (Figs 6b and 7b). At this stage, the animal was lethargic, plasma testosterone, at its minimum, amounted to 0.05 ng/ml and plasma thyroxine, at its peak, to 10.6 ng/ml. Figure 7a also shows a gradual decline in thyroxine levels throughout the period covered by the experiment.

DISCUSSION

The experiments reported here clearly show that annual cycles of hibernation, body weight and secretion of plasma testosterone and thyroxine persisted when the environmental temperature cycle was maintained, despite the lack of a photoperiod cycle. Furthermore, the temporal relationships between the four biological rhythms measured corresponded to those observed earlier in a natural environment (Jallageas and Assenmacher, 1983). Accordingly, body weight rose to its annual maximum before entry into dormancy (September), and testosterone and thyroxine cycles were essentially parallel, with a climax in summer once all animals were normothermic and alert. Finally, as in the earlier series of animals housed under outdoor conditions, the minimum body temperature necessary for the seasonal rise in plasma testosterone and thyroxine to occur was about 30°C.

As regards the annual decrease in both hormones, it took place before the external temperature and, even more, the body temperature started to decrease. This drop might therefore be endogenously controlled. However, a few minor differences were noticed between the animals' responses to a natural photoperiod (earlier series), compared to an unchanging photoperiod (present series). These differences included the loss in the present series, of a clearly biphasic body weight cycle—no prominent secondary peak occurred after the vernal arousal—a slightly depressed annual peak of the thyroxine cycle and the significant emergence of an autumn resurgence of thyroxine and sometimes of testosterone immediately before hibernation. Taken together, these results certainly fit and extend earlier findings that in edible dormice, neither the infradian body weight cycles (Mrosovsky, 1977; Melnyk, 1979; Mrosovsky *et al.*, 1980) nor the hibernation cycle (Butschke, 1977) were dependent on annual variations in the photoperiod. In this respect dormice definitely resemble most other hibernators (rev. in Mrosovsky, 1978), although a few have been shown to respond to predominantly photoperiodic cues for the annual timing of body weight and torpor cycles (e.g. Djungarian hamster, *Phodopus sungorus* (Heldmaier and Steinlechner, 1981), and the meadow jumping mouse, *Zapus Hudsonius* (Muchlinski, 1980)) and of sexual cycles (e.g. golden hamster, *Mesocricetus auratus* (Gaston and Menaker, 1967; Reiter, 1973), and Djungarian hamster, *Phodopus sungorus* (Hoffmann, 1973, 1979)). However, it must be stressed that in certain short-term experiments on edible dormice, body weight and molt (König, 1960) as well as torpor (Morrison, 1964) were influenced by artificial manipulations of the photoperiod which may indicate that dormice are, after all, to some extent photoperiodic. Similarly, the modifications in the biological cycles observed here may also be tentatively ascribed to the lack of an annual photoperiodic cycle. Nevertheless, this study undoubtedly points to an annual ambient temperature cycle as the prime environmental synchronizer of the metabolic and endocrine functions measured. When dormice were exposed to a constant cold ambient temperature in addition to an unchanging photoperiod the pattern of the biological parameters recorded changed dramatically, to the extent that no evidence remained of circannual rhythmic variations in body weight, hibernation, plasma testosterone or thyroxine. Thus, dormancy phases of various lengths were spread over the whole year, the total yearly amount of torpor increased, and variations in body weight, plasma testosterone and thyroxine were restricted to irregular short-lived fluctuations spanning a few weeks only. It was puzzling to note that as regards plasma testosterone and thyroxine, these fluctuations no longer displayed regular relationships to each other or for the latter to hibernation; whereas the testosterone cycle continued to occur only in animals with body temperatures above 30°C, plasma thyroxine frequently rose in dormant dormice and fell to minimal levels in alert specimens. This clearly implies that when the photoperiod and cold ambient temperature are constant, fluctuations in thyroxine and testosterone secretion are induced by separate endogenous mechanisms, whereas under environmentally synchronized conditions, the rhythms of both secretions are clearly correlated.

The absence of circannual cycles of body weight and hibernation contrasting with maintained infradian fluctuations of both functions noted here agrees with earlier results for edible dormice kept in a cold room under a 12 hr light–12 hr dark photoperiod (Schaeffer *et al.*, 1975; Scott and Fischer, 1976; Mrosovsky, 1977, 1978; Mrosovsky *et al.*, 1980). In experiments of long duration, Scott and Fischer estimated the mean length of these short hibernation cycles at 6.1 months, while Mrosovsky stated that infradian body weight cycles ranged from 28 to 425 days, with a mean of 162 days and were directly related to the hibernation cycle. In that series, the latter displayed somewhat variable individual patterns, but several dormice hibernated for more than 6 months. On the other hand, a variety of other hibernators have been shown, unlike edible dormice, to maintain circannual cycles in hibernation and body weight when the photoperiod (12 hr light–12 hr dark) and cold temperature are kept constant. Such hibernators include the golden mantled squirrel (*Citellus lateralis*) (Pengelley and Asmundson, 1974), a number of other ground squirrels (rev. in Mrosovsky, 1978), the European hamster *Cricetus cricetus* (Canguilhem, 1977), the chipmunks *Eutamias* and *Tamias striatus* (Heller and Poulson, 1970; Scott and Fischer, 1972), and the meadow jumping mouse *Zapus Hudsonius* (Muchlinsky, 1980).

In general, little information is available about the effect on endocrine cycles of longterm exposure of hibernators to constant photoperiods and cold. The

European hedgehog *Erinaceus europaeus* was shown to maintain circannual testosterone cycles (Saboureau, 1981), and the garden dormouse *Eliomys quercinus* even displayed prolonged testis activity when exposed to cold in spring (Ambid, 1976; Ambid and Berges, 1981). There are also a few studies of endocrine functions in hibernators exposed to an unvarying photoperiod and a constantly warm ambient temperature. In this particular environment, edible dormice were reported to display infradian testis weight cycles related to short body weight cycles (Joy *et al.*, 1980), whereas an endogenous circannual pattern prevailed for the reproductive function in the ground squirrel *Spermophilus lateralis* and the chipmunk *Eutamias* (Heller and Poulson, 1970), as well as for the kinetics of radio-iodine release in the ground squirrels *Ammospermophilus leucurus* and *Spermophilus* spp. (Hudson and Deavers, 1976).

To sum up, the present observations indicate that, unlike certain other hibernating mammals, edible dormice exposed to an unchanging photoperiod and constant cold temperatures lack endogenous machinery capable of generating circannual functional rhythms. This principle was confirmed here for hibernation and body weight, and its validity was extended to testosterone and thyroxine secretion. Under the present conditions, only irregular series of infradian fluctuations were measured in the four functional items studied; among them, testosterone fluctuations seemed to depend on the existence of warm body temperature periods. The clear annual cycles for hibernation, body weight plasma testosterone and thyroxine previously observed in a normal environment (Jallageas and Assenmacher, 1983) may therefore be essentially controlled by external factors, and the present data definitely indicate that the annual temperature cycle is the main synchronizing cue for these specific biological rhythms, both in their relation to the environment and to each other, even though the photoperiod may slightly affect this control. Whether warm temperatures also have a permissive effect on other endogenous rhythmicities is now being investigated.

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METABOLIC RATES OF SUB-ANTARCTIC PROCELLARIIFORMES: A COMPARATIVE STUDY

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Abstract—1. The relationship between metabolic rate and body mass for 10 species of sub-Antarctic Procellariiformes is described by the equation: $M = 382.2\ W^{0.68} \pm 1.45$.

2. The relationship between metabolic rate and body mass for 10 species of sub-Antarctic, two species of sub-Arctic and three species of tropical and subtropical Procellariiformes is described by the equation: $M = 373.6\ W^{0.66} \pm 1.08$.

3. The relationships are comparable to those derived for other groups of non-passerine birds.

INTRODUCTION

The range in body size among members of the avian order Procellariiformes is exceptional, if not unique (Warham, 1975). The smallest stormpetrel is some 500 times lighter than the largest albatross. This feature makes the order ideally suited for studies of size-related parameters, particularly relationships between body mass and metabolic rate. To date, estimates of basal metabolic rate (BMR) in procellariiforms have been based on standard equations relating body mass to energy consumption, derived mainly from studies of terrestrial non-passerine birds (Lasiewski and Dawson, 1967; Aschoff and Pohl, 1970; Kendeigh *et al.*, 1977). However, evidence suggests that the relationship of body mass to metabolism in procellariiforms may differ markedly from these standard equations (Warham, 1971; Grant and Whittow, 1983).

Body temperatures of birds depend on metabolic rate and rate of heat loss (McNab, 1966). Procellariiforms have body temperatures significantly lower than those of other non-passerines (McNab, 1966; Warham, 1971). The low body temperatures of the procellariiforms suggest low BMRs, high rates of heat loss, or both (McNab, 1966). Warham (1971) suggested that the dense waterproof plumage of procellariiforms results in low thermal conductance and, consequently, low rate of heat loss. Their low body temperatures would therefore imply low metabolic rates. Croxall (1982) has further suggested that there might be differences in the relationship of body mass to metabolic rate between the larger surface-breeding petrels and albatrosses, and the smaller nocturnal burrowing petrels. At present, however, there are few empirical data to support these two hypotheses. Direct measurements of metabolic rate of resting procellariiforms have been confined to two species of northern hemisphere stormpetrels, the Forktailed Stormpetrel (*Oceanodroma furcata*) and Leach's Stormpetrel (*O. leucorhoa*) (Iverson and Krog, 1972; Vleck and Kenagy, 1980; Ricklefs *et al.*, 1980), three species of tropical and subtropical petrels, the Wedgetailed Shearwater (*Puffinus pacificus*) (Pettit *et al.*, in Grant and Whittow, 1983), Bonin Petrel (*Pterodroma hypoleuca*) and Laysan Albatross (*Diomedea immutabilis*) (Grant and Whittow, 1983), and one species of sub-Antarctic albatross, the Wandering Albatross (*D. exulans*) (Brown and Adams, 1983).

This paper examines the relationship between body mass and metabolic rate of the above six species and an additional nine species of sub-Antarctic procellariiforms (this study), using oxygen consumption as an index of metabolic rate.

MATERIALS AND METHODS

The study was carried out at sub-Antarctic Marion Island (46°52′S, 37°51′E) during June 1981 to May 1982. Diurnally active species were caught by hand in the field. Nocturnal species were attracted by spotlight at night and caught by hand or removed from burrows. Only individuals not undergoing prolonged natural fasts (preincubation) were used and all birds were released after experimentation. Oxygen consumption ($\dot{V}_{O_2}$) was measured in translucent, airtight metabolic chambers using an open flow-through system. Three different sized chambers were used, each species being placed in the chamber which best accommodated the individual bird without physical restraint but which was small enough to prevent excessive activity.

A pump passed air, drawn from outside the laboratory, through a regulating flow meter before entering the chamber. Air exiting the chamber was passed through a silica gel drying tube, a Rotameter flow meter and a silica gel/Carbosorb/silica gel tube before entering a Taylor Servomex OA570 paramagnetic oxygen analyser. The flow rate was set to produce a drop in oxygen content of between 1 and 2% below that of ambient air, and consequently differed from one species to the next depending on the size of the bird. A thermocouple, inserted into the chamber through a rubber bung, measured chamber temperature. Although chamber temperature could not be controlled, it remained in the range 0–20°C, a temperature span that the birds might experience under natural conditions. During the study, ambient temperatures at Marion Island ranged from −3.9°C to 17.7°C. In order to minimize disturbance no attempt was made to measure body temperature. An initial period of between 45 min and 2 hr was allowed for the birds to settle and for the air in the chambers to equilibrate. Thereafter, readings of percent oxygen, flow rate and temperature were taken at 30 min intervals over 24 hr at normal photoperiod. The oxygen

Table 1. Metabolic rates, temperatures at which metabolic rates were measured and comparison between measured and predicted metabolic rates of 15 species of procellariiforms

Species	Mean body mass (kg)	No. birds	Measured metabolic rate (kJ day^{-1})	Predicted BMR[a] (kJ day^{-1})	Measured MR/ predicted BMR	Mean temperature and range during experiment (°C)	References
Sub-Antarctic species							
Wandering Albatross *Diomedea exulans*	8.130	4	1755 ± 446	1630	1.07	11.6 (2.2–16.0)	Brown and Adams (in press)
Southern Giant Petrel *Macronectes giganteus*	4.780	6	1154 ± 162	1103	1.05	3.8 (0.0–5.8)	This study
Greyheaded Albatross *Diomedea chrysostoma*	3.753	3	735 ± 125	924	0.80	4.9 (1.5–12.4)	This study
Sooty Albatross *Phoebetria fusca*	2.875	4	715 ± 227	760	0.94	4.8 (0.0–8.4)	This study
Whitechinned Petrel *Procellaria aequinoctialis*	1.287	7	545 ± 64	420	1.30	9.1 (3.1–15.5)	This study
Grey Petrel *Procellaria cinerea*	1.014	2	433 ± 51	353	1.23	16.9 (13.7–19.1)	This study
Kerguelen Petrel *Pterodroma brevirostris*	0.315	2	153 ± 15	150	1.02	10.6 (5.8–12.7)	This study
Softplumaged Petrel *Pterodroma mollis*	0.274	2	151 ± 3	135	1.12	13.6 (9.6–17.8)	This study
Salvin's Prion *Pachyptila salvini*	0.165	3	134 ± 9	93	1.45	12.3 (10.5–13.6)	This study
South Georgian Divingpetrel *Pelecanoides georgicus*	0.127	2	85 ± 6	77	1.11	16.6 (11.6–19.7)	This study
Other species							
Laysan Albatross *Diomedea immutabilis*	2.522	4	644	690	0.93	—	Grant and Whittow (1983)
Wedgetailed Shearwater *Puffinus pacificus*	0.388	—	130	158	0.83	(15.0–23.7) —	Pettit *et al.* in Grant and Whittow (1983)
Bonin Petrel *Pterodroma hypoleuca*	0.169	5	109	95	1.15	—	Grant and Whittow (1983)
Forktailed Stormpetrel *Oceanodroma furcata*	0.049	1	55	38	1.45	(19.0–24.0) 20.0	Iverson and Krog (1972)
Forktailed Stormpetrel *Oceanodroma furcata*	0.045	1	39	36	1.09	—	Vleck and Kenagy (1980)
Leach's Strompetrel *Oceanodroma leucorhoa*	0.045	14	56	36	1.70	(25.5–28.0) —	Ricklefs *et al.* (1980)
Leach's Strompetrel *Oceanodroma leucorhoa*	0.042	1	56	34	1.65	(15.0–25.0) 20.0	Iverson and Krog (1972)

[a] Predicted from equation (5.5) of Kendeigh *et al.* (1977) in kJ/day. $M = 349.7\ W^{0.73}$.

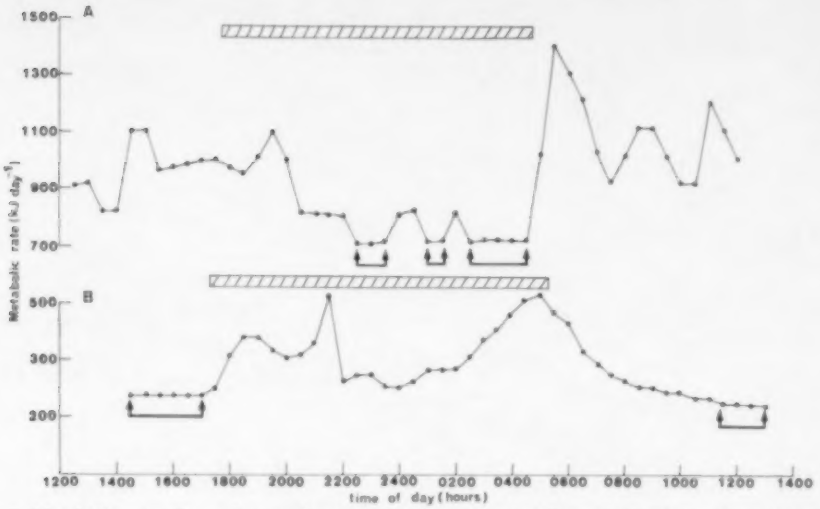

Fig. 1. Metabolic rate of a surface-nesting procellariiform (Sooty Albatross *Phoebetria fusca*) (A) and a burrowing procellariiform (Kerguelen Petrel *Pterodroma brevirostris*) (B) over 24 hr at normal photoperiod. Stable periods used to calculate metabolic rate are indicated by arrows. Hatched areas indicate hours of darkness.

content of ambient air was checked at regular intervals throughout the run. $\dot{V}_{O_2}$ (STPD) was calculated using the equation of Hill (1972). Oxygen consumption was converted to an energy equivalent using the relation that 1 l. oxygen = 20.083 kJ. The lowest stable levels of heat production of at least 2 hr duration were determined. Estimates are given to one standard deviation.

RESULTS

The metabolic rates for 10 species of sub-Antarctic procellariiforms are presented in Table 1. Also included are all values available for metabolic rates of other procellariiforms. The mean body masses given are for the experimental birds only. The predicted basal metabolic rates were calculated from the equation (5.5) of Kendeigh *et al.* (1977). The range of temperatures at which measurements of metabolic rate were made varied from 0°C to 12.4°C for the larger surface-nesting birds (> 2.5 kg) and from 6°C to 19.7°C for the smaller burrowing petrels (< 1.5 kg). The nocturnally active burrowing petrels reached their lowest stable metabolic rate during the day (Fig. 1). The diurnally active surface-nesting birds showed a less well defined pattern. Although stable periods generally occurred at night (Fig. 1), some individuals reached their lowest stable period during the day. The differentiation into nocturnally and diurnally active procellariiforms is only valid during the time birds are restricted to land. The relationship between body mass (W) and metabolic rate (M) for 10 species of sub-Antarctic procellariiforms is described by the equation (Fig. 2)

$$M = 382.2\ W^{0.68} \pm 1.45, \quad r = 0.99.$$

The relationship between body mass and metabolic rate for all species presented in Table 1 is described by the equation (Fig. 2)

$$M = 373.6\ W^{0.66} \pm 1.08, \quad r = 0.99$$

where M is metabolic rate in kJ/day and W is body mass in kilograms. The regression for BMR of Kendeigh *et al.* (1977) is included for comparison.

DISCUSSION

Definitions

Previous measurements of the metabolic rate of procellariiforms generally have been referred to as resting metabolic rate (RMR) (Iverson and Krog, 1972; Grant and Whittow, 1983). Although RMR has been clearly defined by Gessaman (1973) the term is widely used to describe the metabolic rate of a resting animal under any given set of experimental conditions, making comparisons between different studies difficult to interpret. In contrast, basal metabolic rate (BMR) is used and defined in a more restricted sense as the rate of energy metabolism of fasting, inactive birds in the zone of thermoneutrality (Kendeigh *et al.*, 1977). The thermoneutral zones of the birds studied were not known.

Thermoneutral zone (TNZ) and lower critical temperature (T_{lc})

The lower critical temperature (T_{lc}) (the temperature below which body temperature cannot be maintained without an increase in heat production) varies with mass according to equation (5.12) of Kendeigh *et al.* (1977). This equation gives a range in T_{lc} from 9.2°C for a 8.13 kg Wandering Albatross to 23.9°C for a 0.042 kg Leach's Stormpetrel. The equation was determined from studies on temperate, terrestrial non-passerine birds. It is likely that sub-Arctic and sub-Antarctic procellariiforms are cold-adapted and consequently have a lower T_{lc} than temperate terrestrial birds. The T_{lc} of the sub-Antarctic King Penguin (*Aptenodytes patagonicus*) is −5°C (Le Maho, 1977; Groscolas *et al.*, 1981) compared with the predicted value of 8.6°C for a 12 kg bird. In addition, there was no significant correlation between ambient temperature and mass specific metabolic rate ($kJ/kg^{0.68}$) for the larger procellariiforms ($r = 0.217$, $P > 0.10$, $n = 17$). This suggests that the larger sub-Antarctic procellariiforms studied were within their TNZ and that the metabolic rates presented represent an estimate of BMR as

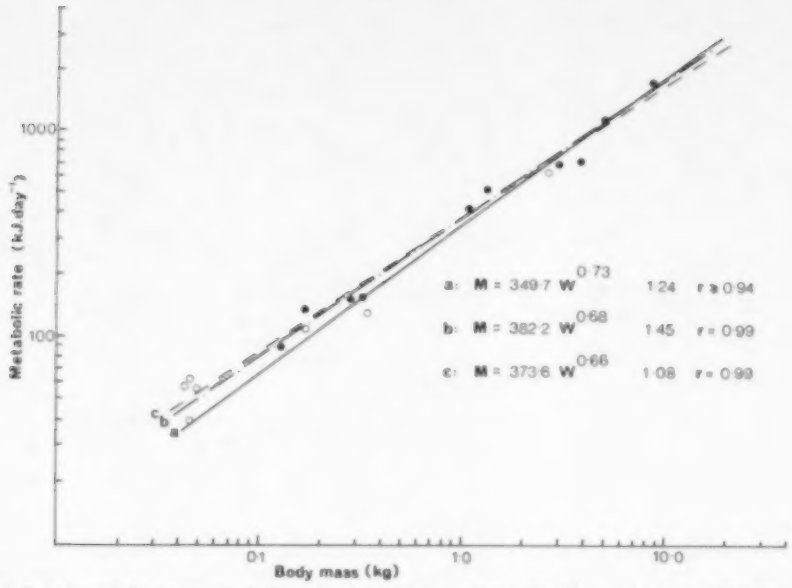

Fig. 2. Relationship between metabolic rate and body mass of procellariiforms. (a) (——): Predicted BMR (Kendeigh *et al.*, 1977). (b) (—·—·—): Sub-Antarctic procellariiforms. (c) (-----): All species listed in Table 1. ●, Sub-Antarctic procellariiforms; ○, non-sub-Antarctic procellariiforms.

defined by Kendeigh *et al.* (1977). However, there was a singificant correlation between body mass and mass specific metabolic rate ($kJ/kg^{0.68}$) for the smaller burrowing petrels but only at the 10% level ($r = -0.456$, $0.1 > P > 0.05$, $n = 18$), suggesting that temperature is having a minor effect on the metabolic rate. Some of the measurements of metabolic rate were probably made below the T_{lc}. These measurements should therefore be regarded as standard metabolic rates (SMRs) that is the metabolic rate of a fasting inactive bird below the TNZ (Kendeigh *et al.*, 1977). Metabolic rates of the non-sub-Antarctic procellariiforms were generally measured at higher temperatures (15.0–28.0°C) (Table 1).

Measured metabolic rates

The larger diurnal surface-nesting procellariiforms had metabolic rates ranging from 20% below to 7% above predicted levels of Kendeigh *et al.* (1977). The metabolic rate of the smaller nocturnal burrowing petrels ranged from 18% below to 70% higher than predicted. Grant and Whittow (1983) consider the metabolic rate (referred to as RMR) of the Laysan Albatross and the Bonin Petrel to be low, consistent with their low body temperatures. However, the measured metabolic rates are 7% below (Laysan Albatross) and 15% above (Bonin Petrel) the predicted BMR (Kendeigh *et al.*, 1977), both within the variation observed for the sub-Antarctic procellariiforms. The metabolic rate of the Wedgetailed Shearwater (Pettit *et al.*, in Grant and Whittow, 1983) is 18% below the predicted value, considerably lower than that measured for other species of burrowing petrels. No details of the experimental procedure are given by Grant and Whittow (1983), therefore the significance of this unusually low value is not clear.

The metabolic rates measured by Iverson and Krog (1972) and Ricklefs *et al.* (1980) for the two stormpetrel species were considerably higher than that obtained by Vleck and Kenagy (1980) for the Forkedtailed Stormpetrel (Table 1). This may reflect differences in experimental procedure. In contrast to the measurements of Vleck and Kenagy (1980) there is no indication that the measurements of Iverson and Krog (1972) and Ricklefs *et al.* (1980) were made over the non-active part of the circadian rhythm. Aschoff and Pohl (1970) have shown that avian BMR averages 20–25% higher during the active phase of the circadian rhythm than during the resting phase. The importance of measuring BMR or SMR during the resting phase and defining this period of inactivity is illustrated in Fig. 1. The dramatic increases in metabolism shown by both birds were related in large part to activity in the chamber that occurred with sunrise or sunset. These activity related increases masked any intrinsic rise in BMR that might be expected to occur in the active phase of the circadian rhythm (Aschoff and Pohl, 1970).

Body mass to metabolism relationship

The regression of BMR to body mass for the 10 species of sub-Antarctic procellariiforms has a slope of 0.68, close to the value of 0.66 for all species of procellariiforms. Croxall (1982) obtained a value of 0.67 from the regression of energy cost of incubation to body mass for procellariiforms, based on mass loss data. The similarity of this figure to the values obtained for this study suggest that the assumptions used in calculating the metabolic rate from the rate of mass loss are essentially correct. Grant and Whittow (1983) obtained a slope of only 0.603 for the regression of RMR to body mass of non sub-Antarctic procellariiforms. This is significantly lower than the value for non-passerines (Kendeigh *et al.*, 1977) ($F_{(1,82)} = 4.82$, $P = 0.03$). However, the equation of Grant and Whittow (1983) is based on only seven values for five

species, of which only one can be considered a large procellariiform (>1.5 kg). The trend from steeper to shallower slopes of the body mass to metabolic rate relationship as proportionally more smaller petrels are included in the regression is due to the elevated metabolism of the smaller petrels. This trend lends weight to the suggestion that the metabolic rates of these birds were measured below the TNZ. The slope 0.68 is not significantly different from the value of 0.73 for non-passerines (Kendeigh *et al.*, 1977) ($F_{(1,85)} = 1.14$, $P = 0.30$).

These results suggest that the BMR of at least sub-Antarctic procellariiforms are generally not lower than those predicted and that they have a body mass to metabolic rate relationship similar to those of other non-passerines. Furthermore, the high correlation coefficient ($r = 0.99$) suggests that there is no difference in the body mass to metabolic rate relationship between the albatrosses and smaller petrels, contrary to the suggestion of Croxall (1982).

Thermal conductance and body temperature

The hypothesis that procellariiforms have low thermal conductances and consequently low rates of heat loss (Warham, 1971) appears to be incorrect. Iverson and Krog (1972) calculated the thermal conductances of two species of stormpetrel to be 55% higher than predicted for several similarly sized species. Likewise, published values for thermal conductance in penguins, which have similar body temperatures to procellariiforms and metabolic rates comparable with predicted values, are higher than might be expected (Stahel and Nicol, 1982). The thermal conductance of even the extreme, cold-adapted Emperor Penguin (*Aptenodytes forsteri*) is not especially low (Le Maho *et al.*, 1976). Therefore, there is no reason to suspect that the larger procellariiforms have abnormally low thermal conductances.

There is, as yet, no satisfactory physiological explanation for the low body temperatures of procellariiforms. Stahel and Nicol (1982) suggested that the low body temperatures recorded for penguins may be due to site of measurement. Differences of between 0.6°C and 5°C have been recorded for simultaneous measurement of stomach and cloacal temperatures. However, Warham (1971) found no significant difference in temperatures measured in the proventriculus and the cloaca of procellariiforms. Iverson and Krog (1972) attributed the elevated metabolic rates and low body temperatures of small petrels to adaptations for maintaining a constant body temperature under varying degrees of cold stress.

Conclusion

This study highlights the need for further work on the effect of temperature on metabolic rate. Closer control of experimental temperature, particularly for the smaller procellariiforms, may eliminate some of the variability in measured metabolic rates.

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ISOLATION AND CHARACTERIZATION OF CAMEL PEPSINS

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Abstract—1. Camel (*Camelus dromedarius*) pepsins were precipitated from the extract of the fundic gastric mucosa by ammonium sulfate between 35 and 80% saturation. DEAE—cellulose chromatography of this fraction produced two isoenzymes, I and II, which were further purified to homogeneity on sephadex G-100.

2. Their K_m with *N*-acetyl-L-phenylalanyl-diiodotyrosine were 0.10 and 0.09 mM, and their molecular weights which were determined on sodium dodecylsulfate gel electrophoresis exhibited 35,500 and 34,700, respectively.

3. The two pepsins were essentially free of carbohydrates, but contained 0.3 and 1.0 mol of organic phosphate per 1 mol of protein, respectively. The apparent mobilities of the phospho- and dephosphoforms of each pepsin were indifferent in polyacrylamide gel electrophoresis at pH 8.9.

4. The N-terminal residues of pepsins I and II were found to be alanine and leucine, respectively.

5. Pepsin I was inactivated at a faster rate than that of pepsin II at pH 8 and 0°C, and at pH 7.5 and 37°C; but both were denatured under these conditions. The properties of these enzymes are compared with those of other mammalian and avian pepsins.

INTRODUCTION

Pepsins are gastric proteases active at pH 1–5, and synthesized as catalytically inactive pepsinogens. Northrop (1930, 1933) isolated and crystallized both pig and bovine pepsins. Multiple forms of porcine (pig) pepsin were reported by Ryle and Porter (1959) and Ryle (1966). Rajagopalan *et al.* (1966) developed a method for the fractionation of commercial crystalline porcine pepsin, and its primary structure has been worked out by Tang and co-workers (Tang, 1970; Tang and Hartley, 1970; Tang *et al.*, 1973; Chen *et al.*, 1975; Marciniszyn *et al.*, 1975; Sepulveda *et al.*, 1975). Bovine pepsinogen was isolated and characterized by Chow and Kassell (1968), and Fox (1969) partially purified its pepsin. Meitner and Kassell (1971) and Lang and Kassell (1971) isolated a number of minor bovine isoenzymes. Nevaldine and Kassell (1971) purified bovine pepsinogen and pepsin on poly-L-lysine covalently attached to sepharose 4B. The amino acid composition and properties of bovine pepsin (Lang and Kassell, 1971) were found different from those of porcine pepsin. O'Leary and Fox (1975) developed a general method for the isolation of pepsins and reported on properties of sheep pepsin (O'Leary and Fox, 1973; Fox *et al.*, 1977). The molecular heterogeneity of human pepsin has been reported (Etherington and Taylor, 1967, 1969; Turner *et al.*, 1970; Whitecross *et al.*, 1974; Roberts and Taylor, 1978). Human pepsin and gastricsin (pepsin C) have been characterized by Mills and Tang (1967) and Huang and Tang (1970). Five pepsinogens and their corresponding pepsins, I, II, III-1, III-2, and III-3, were isolated from Japanese Monkey by Kageyama and Takahashi (1976a,b). The major isoenzyme was III-3. The multiple isoenzymes were then reported in mouse (Esumi *et al.*, 1978), in canine (Marciniszyn and Kassell, 1971), in chicken (Levchuk and Orekhovich, 1963; Bohak, 1969; Donta and Van Vunakis, 1970; Green and Llewellin, 1973), in dogfish (Merrett *et al.*, 1969), in Japanese quail (Esumi *et al.*, 1980), in rat (Muto and Tani, 1979), in crab-eating monkey (Kageyama and Takahashi, 1980a), and in cow (Antonini and Dumas, 1971). Recently, Kageyama and Takahashi (1980b,c) studied the molecular evolution of pepsinogen and applied it to study the phylogenic relationship among animals which possess pepsinogens. This and the uncertainty of the role of the carbohydrates in pepsinogen, the organic phosphate in pepsin A, (the major pepsin) and the presence of the sulfhydryl group, so far, only in chicken system (Bohak, 1969; Donta and Van Vunakis, 1970; Green and Llewellin, 1973) motivated us to isolate, characterize, and compare camel pepsins with those known pepsins. Furthermore, the camel is considered a vanishing species. It, which belongs to the suborder *Tylopoda*, is like other ruminants in that it has rumen, reticulum and abomasum, but not omasum, the third stomach.

MATERIALS AND METHODS

Preparation of camel mucosae

The third stomach of the adult camel (*Camelus dromedarius*) was removed within 30 min after its slaughter. Abomasa were turned inside out and washed thoroughly with cold water. The glandular fundic portions were cut out, the mucosae were dissected from the muscular stomach, and the fatty materials were removed. The mucosae were quick-frozen in liquid nitrogen and stored at −20°C when they were not used immediately.

Chemicals

Porcine pepsin (E.C. 3.4.4.1), diethylaminoethyl-cellulose (DEAE-cellulose), bovine pancreatic chymotrypsinogen A and trypsin (E.C. 3.4.4.4), whale skeletal muscle myoglobin,

*Data are taken in part from a thesis submitted to the Graduate Faculty of the University of Jordan in partial fulfilment of the requirements for the degree of Master of Science.

and *p*-nitrophenylphosphate were purchased from Sigma, Saint Louis, MO. Sephadex G-100 was a product from Pharmacia, Uppsala 1, Sweden. Crystalline bovine serum albumin, acid phosphatase from wheat germ, alkaline phosphatase from porcine intestinal mucosa, and Japanese agar were from British Drug House, London. Dinitrophenyl-amino acids as standards were prepared in this laboratory, human hemoglobin (lyophilized powder) was kindly supplied by Professor R. J. Peanasky, Division of Biochemistry/Physiology and Pharmacology, School of Medicine, the University of South Dakota, Vermillion, SD. Milk powder was from local grocery store. All other chemicals were reagent grade, and glass distilled water was used throughout.

Localization of pepsin activity on agar gel

The method of Uriel (1960) as modified by Etherington and Taylor (1969) was used.

Milk-clotting assay for pepsin activity

The method of Chow and Kassell (1968) was used to assay for pepsinogen–pepsin transformation.

Hemoglobin assay for pepsin activity

The method of Anson (1938) as described by Abu-Erreish and Peanasky (1974) was routinely used to measure the peptic activity through this work. The extent of digestion of hemoglobin by pepsin was determined by measuring the formation of trichloroacetic acid–soluble material as increase at 280 nm. The specific activity of camel pepsin was expressed as μg-equivalent of porcine pepsin per one unit of extinction coefficient at 280 nm (=E) of camel pepsin solution. This was computed from a standard curve on porcine pepsin solution. The concentration of the enzyme was calculated from the relationship, μg of porcine pepsin/ml $= A_{280\,nm} \times 676$. The value 676 is obtained from the molar extinction coefficient of porcine pepsin in 0.1 M sodium acetate buffer, pH 5.3, and its mol. wt, as $\varepsilon = 51{,}700$ and 35,000 daltons, respectively (Blumenfeld *et al.*, 1960).

Protein determination

At the initial steps of purification of camel pepsins, protein was determined by the biuret method (Garnall and Bardawill, 1949). Protein eluted from chromatographic columns was monitored at 280 nm.

Assay of phosphatases

Acid and alkaline phosphatases were determined according to Bessey *et al.* (1946).

Determination of carbohydrates

Carbohydrates were determined as glucose and mannose by the anthrone method (Graff *et al.*, 1951).

Determination of organic phosphate

Proteins were digested in 5 M H_2SO_4 solution, and the phosphate released was determined by the method of Chen *et al.* (1956) as modified by Clement *et al.* (1970).

Dephosphorylation of pepsin

Removal of the phosphate from pepsin by acid phosphatase was performed as described (Meitner and Kassell, 1971).

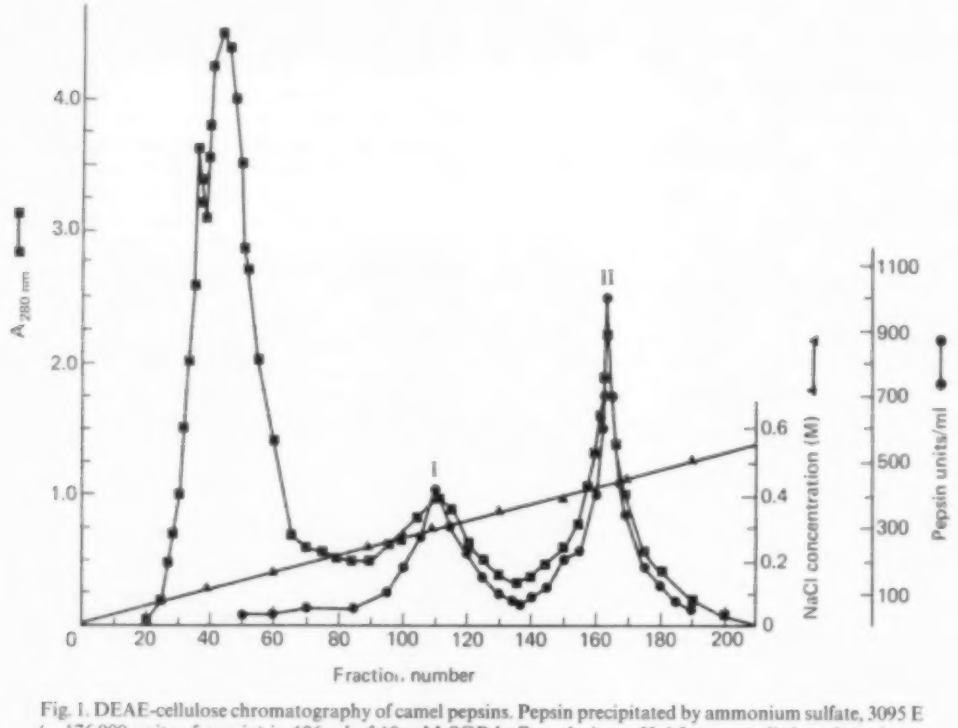

Fig. 1. DEAE-cellulose chromatography of camel pepsins. Pepsin precipitated by ammonium sulfate, 3095 E (=176,000 units of pepsin) in 106 ml of 10 mM SGP buffer solution, pH 6.5, was applied to the column (2.6 × 30 cm) which was equilibrated with the same buffer at a rate of 10 ml/hr. After the protein solution had just passed into the resin, elution was continued with the same buffer. As soon as the absorbance at 280 nm dropped to the base-line (position 0), the elution was continued with a two chambers–gradient system in which the chambers contained 1 l. each of 0 and 1.0 mol of NaCl/l in the same buffer. The fractions were collected, 4.5 ml each. Left ordinate, ■—■ is $A_{280\,nm}$; right ordinate, ●—● is pepsin units/ml; ▲—▲ is NaCl, mol/l.

Disc gel electrophoresis was carried out according to Ornstein and Davis (1964).

Molecular weights were estimated in 10% polyacrylamide gels in sodium dodecylsulfate according to Weber and Osborn (1969) as modified by Dunker and Reukert (1969).

N-terminal analysis was performed according to Sanger and Tuppy (1951), and Sanger and Thompson (1953).

Isolation of camel pepsins

All subsequent operations were carried out in a cold room at 4°C unless otherwise indicated.

Step 1. (A) *Preparation of mucosal crude extract.* Pepsinogen was extracted from the abomasa of adult camels as described (O'Leary and Fox, 1975) with minor modifications. The mucosa (350 g) was ground and homogenized with 350 ml of water. The homogenate was centrifuged in a Sorvall RC-5 angle superspeed, refrigerated, and centrifuged at 15,000 rpm for 30 min. The extract was filtered through a glass-centered funnel to remove the upper slimy layer.

Step 1. (B) *pH fractionation.* This step consisted of three treatments: (a) Pepsinogen was activated to pepsin by lowering the pH of the extract to 2 with 0.5 M HCl solution. The time required for complete activation of pepsinogen at 14°C was determined. The production of pepsin was measured by the milk-clotting method at pH 5.3 (Chow and Kassell, 1968). The extract was left under this condition for 10 min, and was then centrifuged at 15,000 rpm for 30 min. (b) The pH of the supernatant was raised to 5.3 with 0.5 M NaOH solution, and centrifugation was repeated as before. (c) Sodium glycerophosphate–HCl (SGP) buffer, pH 6.5, was added to the clear supernatant in step 2(B) to make the final concentration of SGP 0.01 M, pH 6.5.

Step 2. *Ammonium sulfate fractionation.* Solid ammonium sulfate (AS) was added to the solution in step 2(c) at 0°C to make 35% saturation (Noda and Kuby, 1957). The pH was maintained at 6.5 by intermittent addition of 0.1 M NaOH solution. The suspension was centrifuged at 15,000 rpm for 30 min, and AS was then added to the supernatant to bring the saturation to 80%. The centrifugation was repeated and the pellet was dissolved in 55 ml of 10 mM SGP buffer solution, pH 6.5, and dialyzed against 6 l. of the same buffer solution. The precipitate was removed by centrifugation, and the supernatant was applied to a column of DEAE-cellulose.

Step 3. *Chromatography on DEAE-cellulose.* A column (2.6 × 30 cm) of DEAE-cellulose was equilibrated with 10 mM SGP buffer solution, pH 6.5. The solution of pepsin was passed into the column, and elution was continued until the absorbance at 280 nm dropped to 0.01 or less. A linear gradient of 0 to 1.0 mol/l of NaCl in the same buffer was then applied. Fractions, 4.5 ml each, were collected. The fractions which contained peptic activity were pooled, dialyzed against the same buffer solution, and concentrated against polyethyleneglycol (carbowax) to 8 ml. The fraction was then applied to a column of Sephadex G-100.

Step 4. *Chromatography on Sephadex* G-100. A column (1.8 × 90 cm) of Sephadex G-100 was equilibrated with 0.1 M KCl in SGP buffer, pH 6.5. Pepsin solution from step 3 was passed into the column and elution was continued with the KCl–SGP solution. Fractions, 4.5 ml each, were collected. Peptic fractions as one peak were combined, dialyzed, and concentrated as in the previous step.

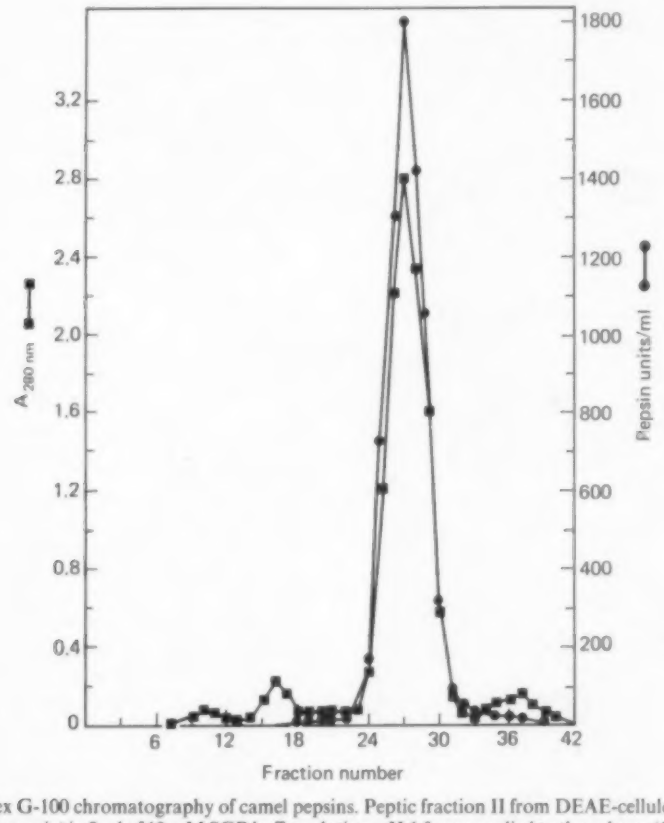

Fig. 2. Sephadex G-100 chromatography of camel pepsins. Peptic fraction II from DEAE-cellulose column, 74.2 E (= 30 mg pepsin) in 9 ml of 10 mM SGP buffer solution, pH 6.5, was applied to the column (1.8 × 90 cm) which was equilibrated and eluted with the same buffer including 0.1 M KCl at a rate of 10 ml/hr. Fractions, 4.5 ml each, were collected. ■—■ is $A_{280\,nm}$; ●—● is μg pepsin/ml.

RESULTS AND DISCUSSION

Step 1. Isolation of pepsin from camel stomach

The time required for activation of pepsinogen to pepsin at pH 2 was initially determined on the crude extract. The results show that the activation of the zymogen was complete in 7 min. The peptic activity in the stomach was found mainly in the fundic mucosa of the abomasum, and it was 8 times that in the pyloric region (i.e. 5.1 μg vs 0.6 μg per mg protein). The total pepsin extracted from a single mucosa (350 g) was equivalent to 130 mg. More pepsinogen was extracted by rehomogenization, but it complicated the procedure of purification of the enzyme by extraction of more nonpepsinogen proteins (the specific activity dropped to 1.9 μg pepsin per mg protein).

Step 2. Precipitation of pepsin by ammonium sulfate

AS at 35% saturation precipitated 67% of the protein in the extract (=5012 mg from the biuret method) with no considerable peptic activity. Pepsin was precipitated by raising the saturation of AS to 80%. The overall recovery of the peptic activity at this step is 67% and the specific activity is 57.

Step 3. Chromatography on DEAE-cellulose

Figure 1 presents the results of the application of the peptic fraction from step 2 (4116 mg protein = 3089E) and its elution from a column of DEAE-cellulose. Two active peaks, I and II, were eluted at 0.3 and 0.4 M NaCl, and they contained 46.4 and 60.0 mg of pepsin, respectively. No further protein could be eluted at 1.0 M NaCl solution, and the protein charged into the column was recovered quantitatively. The fractions 96–130 and 145–185 (specific activity = 200 or more) were combined and labeled pepsin I and pepsin II, respectively. Fraction I contained 38 mg (=115 E) and fraction II contained 50 mg (155 E) of pepsin. The two solutions were dialyzed against SGP buffer and were concentrated as mentioned in the method. The specific activities of the two fractions were 473 and 444, respectively.

Step 4. Chromatography on Sephadex G-100

The results from this step are presented in a typical chromatogram (Fig. 2) for camel pepsin II. Ninety-six per cent of pepsin I (=28 mg) and 92% of pepsin II (=30 mg) were eluted from the column. The active fractions with specific activity of 590 or more were pooled, dialyzed against water, and concentrated as mentioned in the method. The recoveries of the two enzymes amounted to 8 and 9 mg, respectively.

The degree of purity of each pepsin is judged from polyacrylamide gel electrophoresis as shown in Fig. 3; only one band for each enzyme could be observed.

Separation of acid and alkaline phosphatases from pepsins

The presence of phosphatases in the preparation of pepsins might have been possibly responsible for generating the multiple forms of pepsins with respect to the contents of the phosphate.

Both phosphatases were initially present, but later were removed by DEAE-cellulose. Whether they participate in removal of the phosphate from the pepsinogens and pepsins is under investigation. To minimize the dephosphorylation of the zymogens and pepsins by these phosphatases β-glycerophosphate was used in the purification steps as competitive substrate.

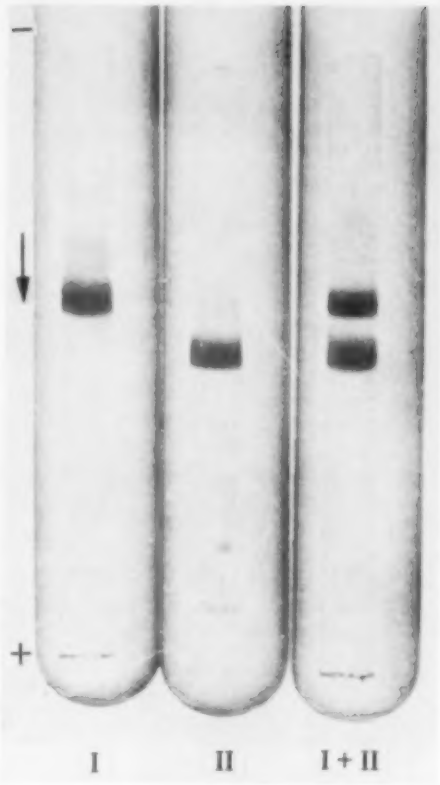

Fig. 3. Polyacrylamide gel electrophoresis. Camel pepsins from Sephadex G-100 column, approximately 80 μg each, were applied to 7.5% gel at pH 8.9. Electrophoresis was performed at 2 Ma/gel for 2 hr in Tris–glycine buffer, pH 8.9, at 0°C. The gels were first fixed in 5% trichloroacetic acid solution and then stained in 5% Coomassie Brilliant Blue solution. The gels were then destained in 7% acetic acid solution in 5% methanol. The proteins migrated towards the anode.

Molecular weights

Pepsins I and II exhibited the same elution volume from Sephadex G-100 column, and the correspondent molecular weight is 37,200. In 10% polyacrylamide gel in the presence of sodium dodecylsulfate, the two pepsins exhibited molecular weights 35,500 and 34,700, respectively (Fig. 4). These values are comparable to those of human (Tang *et al.*, 1967), ovine (Fox *et al.*, 1977), Japanese monkey (Kageyama and Takahashi, 1976), mouse (Esumi *et al.*, 1978), porcine (Rajagopalan *et al.*, 1966), and bovine (Fox *et al.*, 1977). The two camel pepsins, I and II, in acetate buffer, pH 5.3, have molar absorption coefficients at 280 nm as 52,418 and 48,330, respectively, when porcine pepsin was used as a standard; and these figures are in good agreement with those obtained from the amino acid analysis (unpublished data).

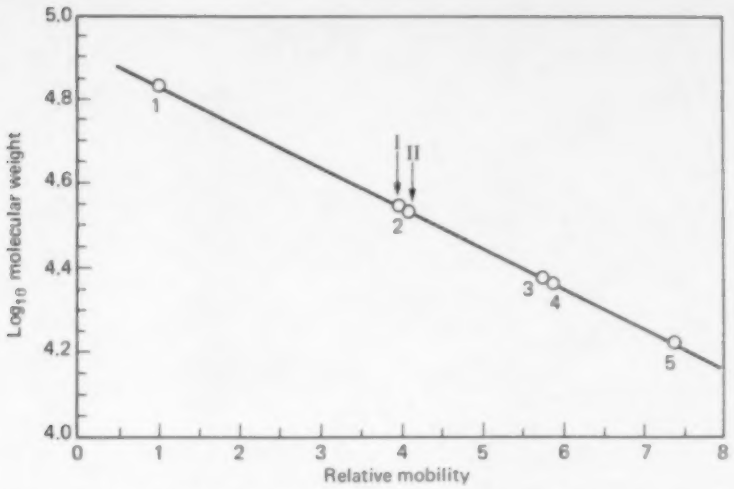

Fig. 4. Determination of the molecular weights in 10% polyacrylamide gel electrophoresis was carried out in sodium dodecylsulfate at pH 7.2. The numbers on the traces refer to : I and II camel pepsins (1) bovine serum albumin, (2) porcine pepsin, (3) bovine chymotrypsinogen A, (4) bovine trypsin, (5) myoglobin.

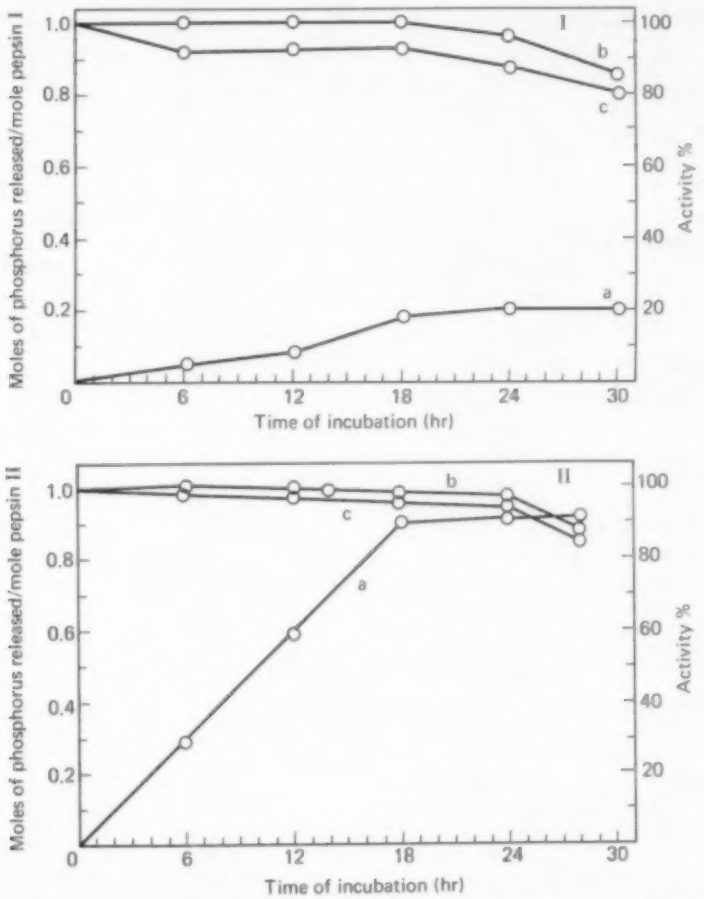

Figure 5. Dephosphorylation and its effect on camel pepsins. To each of 2.5 ml solution of pepsin I and II at 4.5 mg/ml, 1.0 mg of acid phosphatase was added. The two solutions with their controls (minus acid phosphatase) were incubated at 37°C. At 6 hr-interval, a 0.2 ml aliquot was removed and added to 0.4 ml of cold 10% trichloroacetic acid solution. The mixture was heated at 60°C for 15 min, cooled in ice, and centrifuged at 20,000 rpm for 15 min. The phosphate released was then determined. Upper and lower profiles present pepsin I and pepsin II, respectively. (a), Mol of phosphate released per 1 mol of enzyme; (b) and (c), residual activities of the pepsins in the absence and presence of the phosphatase, respectively.

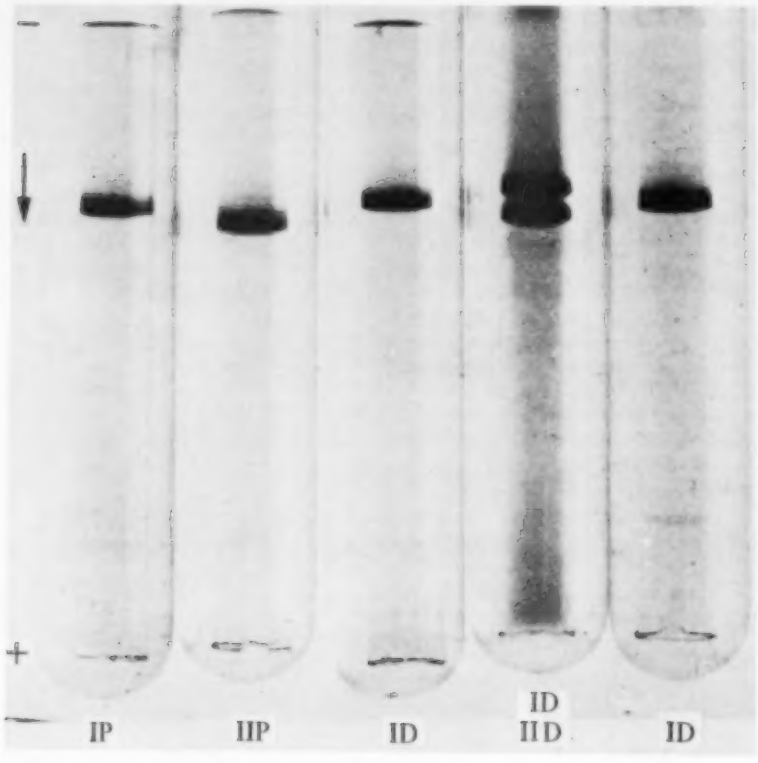

Fig. 6. Polyacrylamide gel electrophoresis of camel dephospho-pepsins. Approximately 50 μg of each enzyme were applied to each of 7.5% gel, at pH 8.9. Electrophoresis and gel staining and destaining were performed as in Fig. 4. The thin bar in the lower part of the gel is the band of bromophenol blue, the tracking dye. I-p and II-p are phospho-pepsins, I and II; I-D and II-D are dephospho-pepsins, I and II, respectively. The proteins migrated towards the anode.

Carbohydrates

No detectable carbohydrates were found in camel pepsins. In this aspect they are similar to porcine (swine) pepsins (Neumann *et al.*, 1969), to chicken pepsins (Bohak, 1969), to canine pepsins (Marciniszyn and Kassel, 1971), to bovine (Fox *et al.*, 1977; Meitner and Kassell, 1971), and to Japanese monkey (Kageyama and Takahashi, 1976). It was suggested that the carbohydrates in pepsinogen is that they stabilize the zymogen in a conformation that protects its active site (Neumann *et al.*, 1969). The suggestion was based on that in the process of activation of pepsinogen to pepsin the sugar moieties were released first, and pepsin contains no carbohydrates. This role of carbohydrates in pepsinogen has been questioned since the finding of Japanese monkey pepsins I and II (and their pepsinogens) contain carbohydrates, and Japanese monkey pepsinogen III (Kageyama and Takahashi, 1976) and camel pepsinogens (unpublished data) lack them.

Organic phosphate

The digestion of camel pepsins, I and II, with H_2SO_4 solution released 0.39 and 1.19 mol of phosphate per mol of protein, respectively. Dephosphorylation of camel pepsins and porcine pepsin by acid phosphatase yielded 0.21, 0.90, and 0.81 mol of phosphate per mol of protein, respectively. The time course of the phosphate

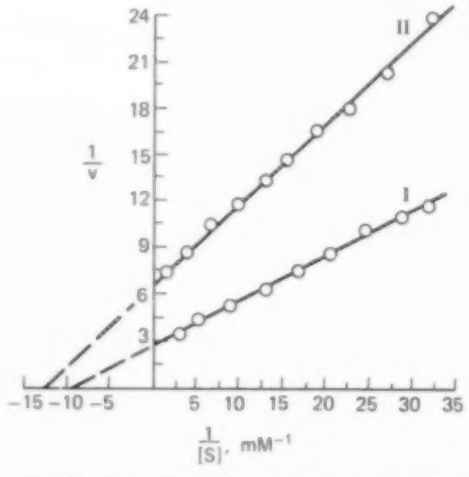

Fig. 7. Lineweaver–Burk plots representing the effects of *N*-acetyl-L-phenylalanyl-L-diiodotyrosine concentrations on camel pepsins activities. The substrate was added to 6 μg of pepsin in 0.25 ml of 0.07 M HCl solution at 37°C, and incubation at this temperature was maintained for 20 min, followed by addition of 1.0 ml of 2.5% ninhydrin solution. The reaction mixture was then boiled (in boiling water) for 15 min, cooled in a tap water, and finally diluted with 3.0 ml of 60% (v/v) ethanol. The color produced was then measured at 570 nm. The plots of reciprocal substrate concentration versus reciprocal velocity was constructed. I and II refer to pepsins I and II, respectively.

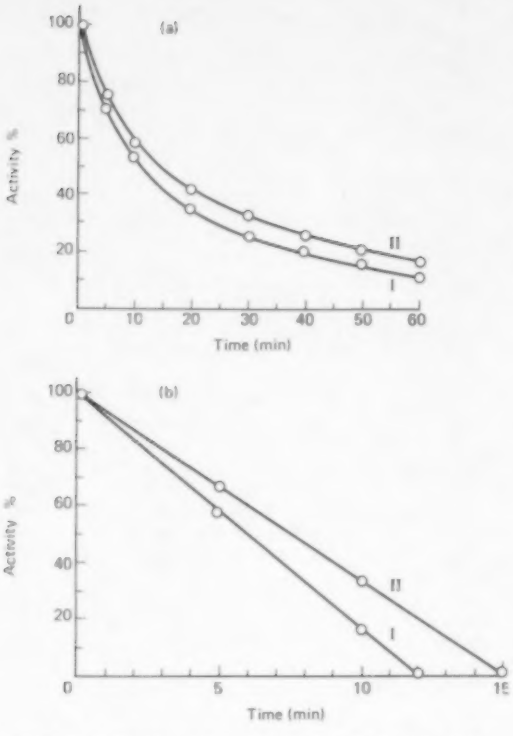

Fig. 8. Stability of camel pepsins under alkaline conditions. (a) solutions of camel pepsins were made in 0.05 M Tris–HCl buffer, pH 8, at 0°C. (b) Solutions of the enzymes were made in 0.05 M Tris–HCl buffer, pH 7.5, at 37°C. I and II refer to camel pepsins I and II, respectively.

release from these pepsins is shown in Fig. 5. These results suggest that pepsin II is the major one which corresponds to pepsin A. Apparently, the electrophoretic mobilities of the dephospho- and the phosphoforms of camel pepsins are indifferent (Fig. 6).

Activities of camel pepsins towards *N*-acetyl-L-phenylalanyl-L-diiodotyrosine-A Lineweaver–Burk plot (Lineweaver and Burk, 1934) of the data obtained from the hydrolysis of this substrate by the two enzymes, I and II, yielded Michaelis constants, K_m of 0.10 and 0.09 mmol/l, respectively (Fig. 7).

N-Terminal residues

The reaction of Sanger's reagent, 1-fluoro-2,4-dinitrobenzene, with camel pepsins followed by acid hydrolysis and identification of the dinitrophenyl amino acids produced dinitrophenylalanine from pepsin I, and dinitrophenylleucine from pepsin II. Having two termini, Ala and Leu, means that the two pepsins are not likely to be one form.

Stability of the camel pepsins to alkaline pH

Figure 8 shows the residual activity of each pepsin as a function of time of incubation at pH 8 and 0°C. Under these conditions, 50% of the initial activity of each pepsin was lost in 10 min. Pepsin II appears more stable than pepsin I. However, at pH 7.5 and 37°C, both pepsins were completely inactivated in 12 and 15 min, respectively. These results all together indicate that the two enzymes are isoenzymes. They have the same optimal pH, 1.8, for hydrolysis of hemoglobin, and the same milk-clotting time. The reason for the partial phosphorylation of pepsin I could not be precisely explained. However, it could be a mixture of two proteins, one is phosphorylated and one is not; or it could be contaminated with non-organic phosphate.

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OXYGEN STORAGE CAPACITY AND TOLERANCE OF SUBMERGENCE OF A NON-AQUATIC REPTILE AND AN AQUATIC REPTILE

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Abstract—1. The oxygen storage capacities and the tolerance to submergence of an aquatic snake, *Natrix sipedon*, and a non-aquatic snake, *Crotalus viridis*, were determined and compared.

2. *C. viridis* was found to have a larger oxygen storage capacity, hemoglobin content and blood volume than *N. sipedon*.

3. The submergence time for *C. viridis* was 30.13 min which was less than the estimated time for submergence based on the oxygen storage capacity.

4. *N. sipedon* exceeded its estimated dive time of 24.00 min and remained submerged for 65.57 min without signs of stress.

5. During submergence, the heart rate of *N. sipedon* fell to 9% (5 beats/min) of the resting rate, while the heart rate of *C. viridis* fell to only 75% (27 beats/min) of the resting rate during this activity.

6. These data indicate that *N. sipedon* responds to submergence via a typical diving reflex and extends its oxygen stores beyond that expected of a non-diver such as *C. viridis*.

INTRODUCTION

The oxygen storage capacity of diving birds and mammals is generally larger than that found in non-diving animals (Andersen, 1966). The beaver which is renowned for its diving ability is an exception and has a smaller oxygen storage capacity per gram than that found for relatively poor divers such as man (McKean and Carlton, 1977). The increase in oxygen storage capacity found in divers is due primarily to a greater blood volume, hemoglobin content and myoglobin concentration (Andersen, 1966).

Most diving birds and mammals are able to extend their diving time 2 to 4 times longer than would be expected if the animal continued to consume oxygen at its resting metabolic rate until the oxygen stores were completely utilized. This is due to physiological changes which occur upon submergence such as reduced metabolism, vasoconstriction in peripheral tissues, and a large decrease in heart rate (bradycardia). These are collectively called the diving response and function to conserve oxygen for those tissues sensitive to hypoxia, i.e. the brain and heart (Andersen, 1966).

A diving-like response has been observed in every bird and mammal submerged (Irving, 1964). However, in animals not well adapted to an aquatic or semi-aquatic habitat, the intensity of the response is greatly reduced. These animals are not able to prolong a dive beyond that time estimated from their resting metabolic rate and oxygen stores. In fact, these nonadapted mammals, such as man, frequently show a dive time that is shorter than expected because of increased metabolism which results from struggling during submergence (Schmidt-Nielsen, 1979).

The response to submergence has been studied in diving reptiles and is similar to that seen in birds and mammals with the exception that the development of bradycardia is variable depending on the conditions of the dive. In restrained dives aquatic and semi-aquatic snakes, alligators and turtles develop a diving bradycardia, i.e. a large decrease in heart rate (Johansen, 1959; Andersen, 1961; Murdaugh and Jackson, 1962; Ferguson, 1966). The onset of the response can develop quickly (Johansen, 1959; Ferguson, 1966) or gradually (Andersen, 1961; Murdaugh and Jackson, 1962). However, the percent drop in heart rate under restraint is usually greater than that seen in a free dive. In free dives of alligators and aquatic and semi-aquatic snakes the heart rate does not always slow in response to submergence (Smith *et al.*, 1974; Jacob and McDonald, 1976; Heatwolf *et al.*, 1979). However, in all cases when the animal felt threatened, such as when approached by man, the heart rate immediately decreased.

The effect of submergence on a non-aquatic species of reptile has not been reported. Belkin (1963) has shown that reptiles as a group are tolerant to anoxia. In his studies nitrogen was used to induce anoxia and no difference was found between aquatic and non-aquatic species in their ability to survive without oxygen.

Irving (1964) has determined the length of time many mammals and birds can remain submerged. Survival time during submergence has only been determined for a few reptilian species. The length of a dive, however, has not been related to oxygen stores except in one species of sea snake (Heatwolf *et al.*, 1979). Hemoglobin content and hematocrit have been determined for aquatic and non-aquatic species (Wood and Johansen, 1974; Seymour and Webster, 1977) but total oxygen stores of the blood and lungs have not been previously reported.

The purpose of this experiment is to compare the duration and intensity of the diving response and oxygen storage capacity between a non-aquatic and an

aquatic snake. The non-aquatic species used was *Crotalus viridis*, the western rattlesnake, which is found in dry climates and feeds on small terrestrial mammals (Stebbins, 1966). *Natrix sipedon*, the common water snake, was the aquatic species used. This animal spends most of its life in water feeding primarily on amphibians and inactive fish (Ditmar, 1931).

MATERIALS AND METHODS

Crotalus viridis was collected at the Birds of Prey Natural Area in southern Idaho. The animals had been in captivity for over 2 months before the beginning of experimental procedures. Specimens were fed and watered once a month except between November and January when they would not feed. *Natrix sipedon* was obtained from Ray Singleton and Co. of Riverview, FL. Work was begun on these animals 1 week after receiving them. *N. sipedon* had water *ad libitum* during the experimental period and were fed once. All experiments were carried out 1 to 2 weeks after feeding.

Plasma volume, hemoglobin content, hematocrit and lung volume were determined in order to estimate oxygen storage capacity. Oxygen storage was then used to estimate submersion time assuming the animals maintained a resting metabolic rate throughout a dive. Maximum times of submergence were determined and compared to estimated time of submergence as a measure of development of the diving reflex in each specimen. Blood samples were obtained by cardiac puncture of anesthetized snakes. Ketamine (HCl was used to anesthetize *C. viridis* (50 mg/kg; Glenn *et al.*, 1972), while Nembutal was used for *N. sipedon* (25 mg/kg; MacDonald, 1972). Hemoglobin content was determined spectrophotometrically after conversion to cyanomethemoglobin (Davidson and Nelson, 1974). Plasma volume was determined by means of a dye dilution technique (Chein and Gregersen, 1962) using Evan's blue dye (T-1824). Hematocrit was determined after centrifuging the blood in microcapillary tubes. Blood volume was estimated from the plasma volume and hematocrit (Chein and Gregersen, 1962).

Total lung capacity (TLC) was determined according to the method of Tenney and Tenney, 1970. Each snake was opened by making a mid-ventral incision and immersed in water in a graduated cylinder. After immersion the lungs were inflated with water by means of a pipette to a pressure of 10 cm H_2O. The volume increase resulting from this technique was taken as total lung capacity.

Blood volume, hemoglobin concentration and TLC were used to calculate the oxygen stores available to the animal during a dive. Although snakes expire air following submergence (personal observation), it is assumed the animals will dive at TLC and that the lung O_2 concentration is 15% (McKean and Carlton, 1977; Lenfant *et al.*, 1970). Therefore TLX × 0.15 = ml O_2 in the lungs. It is assumed that one-third of the blood volume is arterial and that it is 95% saturated (Lenfant *et al.*, 1970). Snake blood holds 1.34 ml O_2/g hemoglobin (Seymour and Webster, 1977), the O_2 content in arterial blood is 1.34 ml O_2/g Hb × (g% Hb × arterial blood volume) × 0.95. Two-thirds of the blood volume was assumed to be venous with an arteriovenous O_2 difference of 5 vol% (Tucker, 1966). Therefore O_2 content in venous blood = (O_2 concentration in arterial blood − 5 vol%) × ml venous blood. The amount of oxygen dissolved in the tissue fluids is very small and was considered insignificant for this investigation. Myoglobin concentration was not determined as the oxygen stored in the muscles is utilized rapidly and does not aid in prolonging a dive (Andersen, 1966). Total oxygen stores were calculated from the formula: O_2 stores = ml O_2 in lung + ml O_2 in arterial blood + ml O_2 in venous blood. All gas volumes were converted to standard temperature and pressure.

Heart rates were determined in unanesthetized snakes which were taped to a metal rod slightly larger than the diameter of the body. Needle electrodes were inserted subcutaneously lateral to the heart. The electrodes were connected to a high gain amplifier (Narco Bio-Systems type 7171) and electrocardiograms (ECG) were recorded on a chart recorder. This method gave a distinct QRS complex and the average heart rate at various times during a dive could be calculated from the R–R intervals.

After the initial preparation each animal was covered with a cardboard box and left undisturbed for 10–20 min. After this period, resting pre-dive heart rate was recorded and the animal was submerged in water maintained at 20 ± 1°C. The body temperature of each snake equilibrated with the water temperature within 5 min. A dive was continued until the snake either exhaled large quantities of gas from the lungs and/or struggled violently. Struggling always resulted in the loss of the ECG. The length of the dive was recorded and the snake was removed from the water, covered as before and allowed to recover. This time of submergence was taken to be the maximum dive time for the animal. After 2–3 min a post-dive heart rate was recorded for 1 min for each snake. In two cases the animals had dislodged an electrode while struggling and after removal from the water the electrodes were replaced and post-dive heart rates were recorded before 5 min had passed.

Resting metabolic rate for the 2 species at 20°C was estimated from an allometric formula relating metabolic rate to body mass (Bennett and Dawson, 1972). An estimated dive time was calculated by using metabolic rate and the oxygen storage capacity with the formula: estimated dive time = total O_2 stores × 1 hr/ml O_2.

Means of all comparative data were analyzed using Students *t*-test.

RESULTS

The values used to calculate lung and blood oxygen stores for the 2 species of snakes are shown in Table 1. The mean hemoglobin value for *C. viridis* is significantly higher than that of *N. sipedon*. The mean hemoglobin value of 15.5 g/100 ml blood (g%) is greater than that found in other reptiles and is in the range found for this variable in mammals (see Wood and Johansen, 1974). The mean value of 10.3% hemoglobin for *N. sipedon* agrees with the values found by other investigators (Altland, 1971). The blood volume of *C. viridis* is 8.4% of body mass and is significantly greater than the value of 4.8% of body mass found for

Table 1. Values for total lung capacity and blood parameters for *C. viridis* and *N. sipedon* used to calculate oxygen storage capacity

Species	Body mass (g)	Hb (g%)	Hct (%)	Blood volume (% body mass)	TLC (ml)
C. viridis	168.7	15.5*	25.9	8.4*	10.9
±SE	11.6	0.8	1.7	0.7	0.8
n = 8					
N. sipedon	246.5	10.3	24.2	4.8	15.5
±SE	34.1	0.7	1.3	0.3	1.5
n = 9					

*$P < 0.005$ when compared to *N. sipedon*.

All values represent means. SE Standard error, *n* number of species, Hb hemoglobin, Hct hematocrit, TLC total lung capacity.

Table 2. Experimental dive time for *C. viridis* and *N. sipedon* and estimated dive time for the 2 species as calculated for metabolic rate and O_2 stores.

Species	O_2 storage (ml O_2/kg)	Metabolic rate at 20°C (ml O_2/hr)	Estimated dive time (min)	Experimental dive time (min)
C. viridis	21.96*	6.22	35.45	30.13
±SE	1.28	0.49	1.67	6.10
$n = 8$				
N. sipedon	13.91	8.23	24.00	65.57
±SE	1.16	0.87	1.50	9.52
$n = 9$				

*$P < 0.005$ when compared to *N. sipedon*.
All values represent means. Symbols are the same as those given in Fig. 1.

N. sipedon. The blood volume values for *N. sipedon* and *C. viridis* fall in the lower and upper range respectively for reptiles (Altland, 1971). The hematocrit and TLC for both species are not significantly different.

The combined oxygen storage capacity of the blood and lungs is shown in Table 2. *C. viridis* has a significantly greater O_2 capacity than *N. sipedon* due to its larger hemoglobin content and blood volume. The estimated duration of a dive as calculated from the oxygen stores and the resting metabolic rate at 20°C is also shown in Table 2 along with the maximum experimental dive time for *N. sipedon* and *C. viridis*. In *C. viridis* the mean estimated dive time was greater than the mean experimental recorded dive time. *N. sipedon* remained submerged on the average nearly 3 times longer than the estimated dive time of 24 min.

The drop in heart rate from the resting pre-dive heart rate of 36.0 beats/min for *C. viridis* and 57.4 beats/min for *N. sipedon* is illustrated in Fig. 1. *N. sipedon* shows a response expected of a diving species under restraint. The heart rate, within 2 min, had dropped to 36% (20.8 beats/min) of resting. After 15 min the heart rate leveled off to about 9% (5.1 beats/min) of the pre-dive heart rate and remained there for the duration of the dive. In contrast, *C. viridis* showed a drop of only 4% (35.0 beats/min) after 2 min and the heart rate continued to decline slowly throughout the dive to 75% (27.0 beats/min) of the pre-dive heart rate after 20 min. Two individuals of this species remained submerged for over 1 hr with the heart rate dropping to 48% (16.0 beats/min) of the pre-dive level by this time. However, there was not a single case where the heart rate of *C. viridis* decreased to a level comparable to the bradycardia observed in *N. sipedon*. In both species the post-dive heart rate of 26.9 beats/min for *C. viridis* and 43.0 beats/min for *N. sipedon* had only returned to 75% of the pre-dive level after the recovery period.

Experimental dives of both species were characterized by complete relaxation during a dive. Only near the end of a dive did the animal struggle violently. At this time the struggling was intense and the ECG tracings were obscured. After removal from the water the animals were flaccid, often gasping for air, but began struggling after 30–60 sec. It appeared that the rattlesnakes were drinking during the early part of the dive. This behavior occurred in all individuals of this species and in some cases lasted up to the point where struggling began.

DISCUSSION

N. sipedon shows a typical response to submergence seen in most diving animals. Diving animals are able to extend their oxygen stores 2 to 4 times longer during submergence than would be expected in non-aquatic species. *N. sipedon* remained submerged 3 times longer than its estimated time of submergence during experimental dives. As observed in other diving reptiles (Murdaugh and Jackson, 1962; Ferguson,

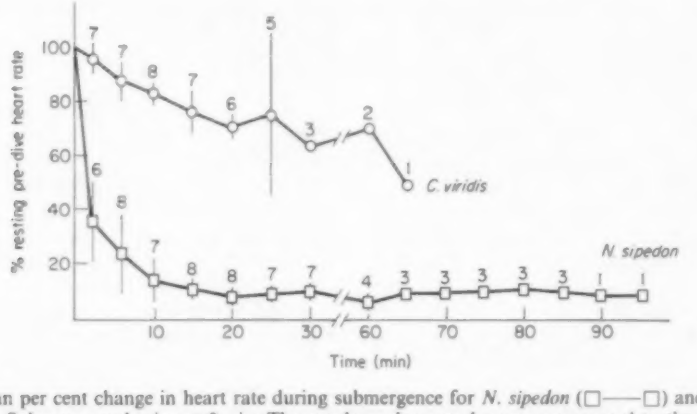

Fig. 1. Mean per cent change in heart rate during submergence for *N. sipedon* (□——□) and *C. viridis* (○——○). Submergence begins at 0 min. The numbers above each curve correspond to the number of snakes for which heart rate was determined at that time interval. The vertial lines represent 95% confidence intervals.

1966), a rapid and intense bradycardia developed in *N. sipedon* soon after submergence and was maintained throughout the dive. It is assumed that peripheral vasoconstriction occurs along with diving bradycardia which conserves oxygen for the brain and other tissues sensitive to hypoxia (Andersen, 1966).

C. viridis reacted to submergence much like non-diving animals and fell 5 min short of its estimated time of submergence during experimental dives. A rapid bradycardia did not develop during the early part of a dive but there was a slow decline in heart rate throughout the dive, a reaction often seen in non-aquatic species (Irving, 1964).

In some species the failure of the heart to show significant bradycardia may be attributed to experimental manipulation during the dive, i.e. restraint. Andersen (1961) found that there was a difference in onset of bradycardia in alligators subjected to involuntary and voluntary periods of submergence. In alligators bradycardia developed more slowly in the involuntary dives; however, minimum heart rate was greater than that seen during voluntary submergence. Murdaugh and Jackson (1962) found that the onset of bradycardia following submergence when the snakes were restrained varied from snake to snake in *N. sipedon* and in *N. cyclopion* but always appeared within 2 min. Bradycardia did not always occur in free dives of snakes and alligators (Jacob and MacDonald, 1976; Smith *et al.*, 1974). However, when the animal was threatened bradycardia developed within 1 heart beat. In this study, *N. sipedon* developed an intense bradycardia within 2 min. However, bradycardia as seen in aquatic species could not be induced in *C. viridis* during submergence.

There are few data on the length of time reptiles can endure submergence. Graham (1974) found that sea snakes could endure over 350 min of submergence depending on the pO_2 of the sea water. These animals are able to utilize cutaneous respiration to acquire up to 33% of their oxygen needs and in free dives probably do not depend on anaerobic metabolism (Graham, 1974). Species of turtles are known to remain submerged for up to 98 min (Belkin, 1964), while snakes of the *Natrix* genus remained submerged for periods up to 30 min during free dives (Smith *et al.*, 1976).

Belkin (1963) has measured the time that several families of reptiles can withstand anoxia. He found a wide range in survival time for the different families and attributes this variability to the wide scope in which reptiles can utilize anaerobic metabolism. Belkin gives no indication of the species he used, however, the estimated dive time of *N. sipedon* falls at the low end of the range of the family Colubridae. When Murdaugh and Jackson (1962) allowed one snake (it is not clear if it was *N. sipedon* or *N. cyclopion*) to breathe pure nitrogen the animal became cyanotic and comatose after 20 min. However, the rapid onset of bradycardia and the significant decrease in heart rate upon submergence and the extended experimental dive time of *N. sipedon* in this study, would indicate that the animal is responding to the water stimulus and not merely the anoxic situation.

The estimated dive time and experimental dive time of *C. viridis* fall short of the anoxic period recorded by Belkin (1963) for Viperidae. However, he only used 3 species that were unfortunately not identified. The similarity between the experimental dive time and estimated dive time supports the hypothesis that the change in heart rate seen in *C. viridis* is due the apneic situation and not the physical stimulus of submergence which plays such an important role in initiating the diving response in birds and mammals (Andersen, 1966).

The usual procedure for determining tolerance to submergence is to submerge the animal and record the time to the last movements, thus drowning the animal (Irving, 1939; Graham, 1974). In this study, however, the animals were removed from the water at the beginning of violent struggling. This struggling was often accompanied by expiration of large volumes of gas from the lungs of *C. viridis*. *N. sipedon* released small amounts of gas throughout the dive but towards the end of a dive in every case the animals would extend their heads toward the surface. This procedure may not give maximal submergence time but it is felt that the times recorded for the 2 species are beyond that occurring in free dives.

The blood volume of *C. viridis* is greater than the 6% of body mass reported for the gopher snake, the only other terrestrial snake for which blood volume has been estimated (Smeller *et al.*, 1978). The blood volume of *N. sipedon* is at the low end of the range of blood volume for reptiles (Altland, 1971) and is lower than the 7% reported for *Hydrophis belcheri*, a marine species, the only non-terrestrial snake for which blood volume has been reported (Seymour and Webster, 1977).

The hemoglobin content of *C. viridis* is higher than that of all other reptiles studied including *N. sipedon* (Altland, 1971). *C. viridis* used in this study is found in a warmer climate than is *N. sipedon*. *C. viridis* feeds mainly on small mammals whereas *N. sipedon* feeds on small fish and amphibians (Ditmar, 1934). The warm climate where *C. viridis* is found would lead to an increase in metabolic rate and oxygen requirements for this species. The oxygen affinity of hemoglobin would be reduced in this warmer climate also. This would facilitate unloading of oxygen at the tissue level but it would inhibit oxygen binding in the lungs. A large supply of oxygen is necessary and hence a higher hemoglobin concentration is found in animals capable of capturing and consuming small rodents.

Diving birds and mammals generally have a greater oxygen storage capacity than their terrestrial relatives due to a greater blood volume and hemoglobin content (Andersen, 1966). The same would be expected in reptiles. However, in this study the aquatic species, *N. sipedon*, has a much lower oxygen storage capacity than does the terrestrial species, *C. viridis*. It is impossible to determine if the trend of greater oxygen stores in diving forms over non-diving forms applies to reptiles as well as mammals and birds due to the dearth of reptilian data on this subject.

The drinking behavior observed during submergence of *C. viridis* may have been an attempt to utilize buccopharyngeal respiration. Aquatic respiration through vascularized buccopharyngeal tissues has been attributed to marine snakes (Ditmar, 1937), and is probably important among marine reptiles since sea water is usually close to oxygen saturation. Fresh waters on the other hand may be deficient in oxygen, and buccopharyngeal respiration would be of limited

use to freshwater species (Graham, 1974). However, Belkin (1968) found that *Sternotherus minor*, a freshwater turtle, could utilize aquatic respiration enabling it to prolong its periods of submergence.

In summary, the response and tolerance to submergence of *N. sipedon* is similar to that of other diving animals whereas *C. viridis* responded as expected for a non-diving animal. However, unlike diving animals, the aquatic snake, *N. sipedon*, did not have a larger oxygen storage capacity than the terrestrial snake, *C. viridis*.

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PHYSIOLOGICAL THERMOREGULATION OF MATURE ALLIGATORS

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Abstract—1. A 67.1 kg alligator (*Alligator mississippiensis*), tested in air, heated twice as fast as it cooled. The cooling thermal time constant was 425 min while alive. Warming and cooling thermal time constants were 421 min after death. The thermal time constant was not appropriate in describing warming in air of mature alligators.

2. Surface and subdermal heat flow measurements of the 67.1 kg animal indicate greater blood flow in the skin during warming compared to cooling.

3. Two mature alligators, 49.9 and 103 kg, were heated and cooled in water. Warming time constants were 67 and 116 min respectively. Cooling time constants were 180 and 246 min.

4. Data from this study were combined with previously published thermal time constants for alligators providing regression equations for alligators ranging from 37 g to 103 kg. Regression equations for alligators tested in water are:

$$\tau_w = 8.81\, M^{0.50}$$

$$\tau_c = 12.6\, M^{0.62},$$

Time constants (τ) are in minutes (w = warming, c = cooling) with all measurements in stirred water; mass, M, is in kg.

5. Thermal conductance and metabolism data are combined to provide an estimate of the amount the body temperature of theoretical alligators ranging from 50 g to 1000 kg would be elevated by metabolism. A body temperature of 34.2°C is predicted for a 1000 kg theoretical alligator in 30°C water.

INTRODUCTION

Thermoregulation has been well studied in crocodilians (Spotila *et al.*, 1972; Spotila, 1974; Lang, 1975, 1976; Smith, 1975a, 1975b, 1976a, 1976b, 1979; Smith *et al.*, 1978; Robertson and Smith, 1979; Smith and Adams, 1978; Johnson *et al.*, 1978; Grigg and Alchin, 1976). From these varied studies of crocodilian thermoregulation, certain features are becoming clear. For example, the importance of thermoregulatory behavior has been well documented. Given the proper environmental parameters and free access to water, large alligators are able to remain remarkably thermostable.

Laboratory studies have indicated the importance of physiological adjustments in altering heat exchange rates. Crocodilians, like many other reptiles (see the early work of Bartholomew and Tucker, 1963, 1965; Bartholomew *et al.*, 1965) heat faster than they cool. They do this by changing the pattern of blood flow in response to temperature changes (Smith, 1976b; Grigg and Alchin, 1976). Many features of this mechanism have been described for other reptiles (Morgareidge and White, 1969; Weathers, 1971; Weathers and Morgareidge, 1971; Baker *et al.*, 1972; see also reviews by Templeton, 1970; White, 1973).

If one defines physiological thermoregulation in terms of the ability to prolong cooling time as opposed to time for warming (Smith, 1979), then it has been found that large alligators are better physiological thermoregulators than small ones (Smith, 1976a; Smith and Adams, 1978; Smith *et al.*, 1978). Measurements and calculations of heat exchange rates based on heating and cooling experiments have shown that small alligators cannot increase cutaneous perfusion in response to warming to the same extent that large alligators can.

Laboratory thermal responses of alligators exceeding 10 kg have not previously been reported. The purpose of this study is to examine the thermal time constant and heat exchange of mature alligators and to provide some generalizations about alligator thermoregulation.

MATERIALS AND METHODS

A 67.1 male *Alligator mississippiensis* was collected on St. Helena Island, Beaufort County, South Carolina on 24 July 1976. The animal was held in a large shaded enclosure containing about 25 cm of water. Experiments began 9 days later. The animal was securely tied to a perforated plywood board and placed in an environmental chamber measuring about 1.5 × 2 × 2 meters. Air flow was parallel to the length of the animal and adjusted to approximately 300 cm/sec. Relative humidity was approximately 48% during warming and 72% during cooling.

Calibrated heat flow discs were used to measure surface and subdermal heat flow. The discs were positioned laterally on the tail as described by Smith (1976b). Output was measured with a portable microvolt meter having an accuracy of ±5%. Temperature measurements were made by Yellow Springs Instrument Company thermistor thermometer, model 43TD, and thermistor (400 series) probes.

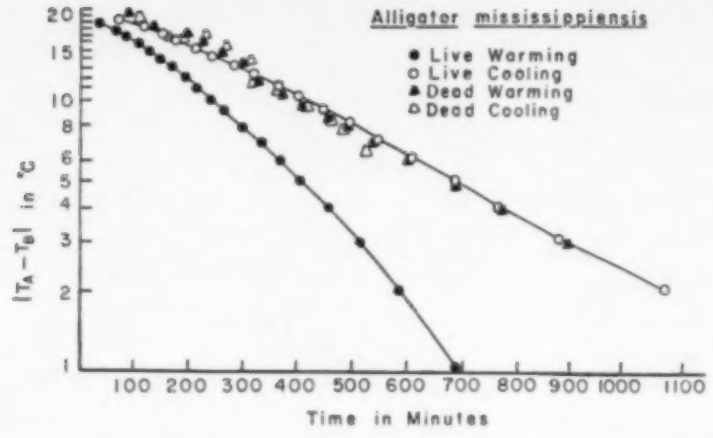

Fig. 1. Heating and cooling response to a live 67.1 kg alligator tested in an air flow of 300 cm/sec.

Temperatures measured included: stomach (via mouth), T_b; cloacal (20 cm), subdermal near the subdermal heat flow disc, surface, T_s; near the surface heat flow disc, head near the skull, deep mid-tail near the caudal vertebrae and air temperature, T_a. All measurements were made at intervals when the stomach temperature was at each whole degree. After cooling to 15°C overnight, with all leads attached, the animal was transferred to a second chamber at 35°C for warming. In subsequent experiments, the chamber temperature was changed instead of moving the alligator. The environmental temperature could be changed in considerably less time than it took for the alligator's body temperature to change 1°C. To minimize error, the first measurement in each experiment was discarded. Unfortunately, the alligator died of undetermined causes after the second cooling experiment. The dead animal was tested and the results compared to the results obtained while alive. The use of data from this animal is justified for the following reasons: (1) few data from mature alligators are available; (2) the thermal response and heat flow measurements are qualitatively and quantitatively similar to published results from smaller alligators; (3) data from dead animals (with no blood flow) provide information useful for understanding living animals.

Two male alligators (49.9 and 103 kg) were collected on the Welder Wildlife Refuge, San Particiao County, Texas. They were held overnight, tested the next day in the laboratory (radiant heating was negligible) and released. Stomach and cloacal thermistors were inserted and the alligators were placed in a 2 m diameter steel tub containing 0.5 m water at 15°C. The temperature was maintained by adding ice as needed. Water was circulated with a small electric trolling motor. Water temperature varied less than 1°C. After the body temperature reached 15°C, the animal was transferred to an identical container containing water at 35°C. Water temperature was maintained by adding hot water. Measurements were made and the time recorded at each whole degree increase in stomach temperature. After equilibrium was reached at 35°C, the animal was again transferred to the cool tub and cooling data collected. Student's *t*-test for paired data was used to test for significance of various temperature differences.

RESULTS

All alligators tested heated faster than they cooled in air and water. The warming thermal time constants for the 49.9 and 103 kg animals were 67.4 and 116 min respectively. Cooling thermal time constants were 179.7 and 246 min respectively. Combining these measurements with previously reported data (Smith, 1975a; Smith and Adams, 1978) yields the following allometric regression formulas for 22 alligators ranging in mass from 37 g to 103 kg tested in water $\tau_w = 8.81M^{0.50}$ and $\tau_c = 12.61M^{0.62}$ (mass, M, in kg and τ in min) respectively for warming and cooling. Correlation coefficients were greater than 0.99.

Thermal time constants for the 67.1 kg alligator were 421 min and 425 min for dead and cooling conditions respectively. The trend for warming cannot be unambiguously characterized by a time constant. The time required for core temperature to reach within 1°C of ambient temperature was half as large during warming as it was for cooling.

Figure 1 shows the heating and cooling response of the 67.1 kg alligator in air. In the air experiment the cloacal temperature lagged behind stomach temperature, especially during cooling. Head and stomach temperatures remained relatively close during warming, but the head cooled faster than the stomach. Tail and stomach temperatures remained close during warming, but the tail cooled faster than the stomach. Temperature measurements were repeated after the alligator died. Mean differences at $T_b = 25$°C during each experiment are summarized in Table 1.

Figure 2 shows the relation between heat flow and the difference between body core and air temperature. Skin surface heat flow during warming exceeded skin

Table 1. Mean temperature differences (°C) at $T_b = 25$°C during warming and cooling of a 67.1 kg American alligator

Temperatures	Condition	Alive	Dead
Stomach – cloacal	Warming	0.73*	0.75*
	Cooling	4.09	−0.26
Stomach – deep tail	Warming	−1.06	−3.66
	Cooling	4.73	3.57
Stomach – head	Warming	0.88	−5.84
	Cooling	3.64	5.84
Stomach – subdermal	Warming	−4.00	5.26
	Cooling	7.82*	7.30*
Stomach – skin surface	Warming	−5.77	−8.32
	Cooling	8.50*	8.32*

The numbers shown are means for 24, 25 and 26°C body temperature.
*Results that do not differ at the 0.01 level of significance between living and dead conditions.

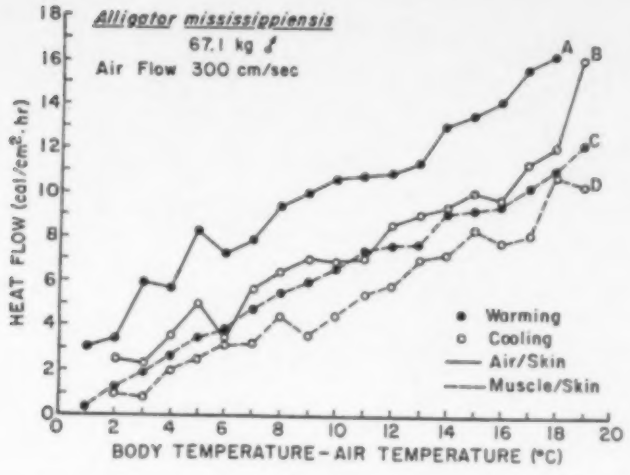

Fig. 2. The relation between heat flow and the difference between core/body temperature and the air temperature for a live 67.1 kg alligator tested in an air flow of 300 cm/sec.

surface heat flow during cooling. Surface heat flow exceeded subdermal heat flow considerably during warming, but only slightly during cooling. Surface and subdermal heat flow measurements were identical within error of measurement for the dead animal.

DISCUSSION

Results obtained for the large animals are qualitatively similar to those previously reported for smaller ones excepting a curvature of the warming lines. Because of the weaker thermal coupling to the environment for animals in air, the curvature is quite pronounced for the 67.1 kg animal (see Fig. 1). The curvature can be most simply attributed to the combined effects of metabolism and changing thermal conductance. See Robertson and Smith (1981) for a discussion of these effects. Evaporative cooling would produce a reversed curvature with body temperature finally equilibrated below ambient temperature.

Thermal conductance was evaluated as $C = H_s/|T_s - T_b|$; H_s being the measured surface heat flow rate. Conductance is shown in Fig. 3 to be both temperature dependent and profoundly different between conditions of warming and cooling. Because subdermal heat flow rates differ little between warming and cooling, whereas surface heat flow rates are markedly higher during warming, one must attribute most of the increased warming conductance to the effect of cutaneous blood flow. Clearance rates for cutaneously injected radioisotopes also support this conclusion (Baker *et al.*, 1972; Grigg and Alchin, 1976; Smith *et al.*, 1978). A more uniform body temperature distribution during warming conditions is shown by the data of Table 1. These data, as well as the markedly increased heart rates during warming, strongly suggest that interior body blood flows are also increased during warming. Besides a lack of other mechanisms to transport the heat required to even out the temperature distribution, it should be noted that the most efficient means of heating the body would be one in which the increased cutaneous flow was matched by an equal increase of interior blood flow.

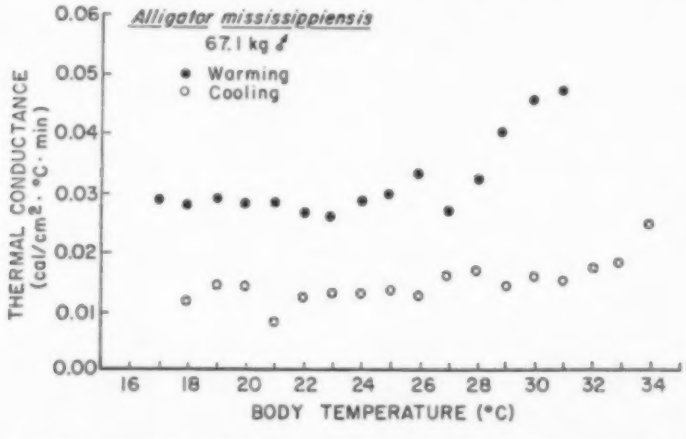

Fig. 3. The relation between area specific thermal conductance and body temperature for a live 67.1 kg alligator tested in an air flow of 300 cm/sec.

Table 2. Thermally significant characteristics of theoretical alligators in 30°C water

Body mass* (kg)	Skin thickness (cm)	τ_w† (min)	τ_c‡ (min)	τ_w/τ_c ratio	Metabolism§ (cal/cm²/min)	Conductance‖ (cal/cm²/min/°C)	$T_b - T_w$¶ (°C)	T_b (°C)
0.05	0.026	1.98	1.99	0.99	0.0194	0.1689	0.11	30.1
0.10	0.033	2.80	3.05	0.92	0.0197	0.1335	0.15	30.2
0.50	0.061	6.24	8.23	0.76	0.0204	0.0772	0.26	30.3
1.00	0.080	8.81	12.6	0.70	0.0208	0.0610	0.34	30.3
5.00	0.147	19.6	34.0	0.58	0.0215	0.0353	0.61	30.6
10.0	0.192	27.7	52.1	0.53	0.0219	0.0279	0.79	30.8
50.0	0.354	61.8	140	0.44	0.0227	0.0161	1.41	31.4
100	0.460	87.3	215	0.41	0.0231	0.0127	1.81	31.8
500	0.849	195	580	0.34	0.0239	0.0074	3.25	33.3
1000	1.104	275	889	0.31	0.0243	0.0058	4.18	34.2

*Skin thickness (S) in cm and mass (M) in kg, $S = 0.080W^{0.38}$ (Smith *et al.*, 1978).

† $\tau_w = 8.810M^{0.698}$, this paper.

‡ $\tau_c = 12.61M^{0.616}$, this paper.

§ Metabolism $= \frac{\text{cooling metabolism}}{\text{area}} = \frac{1.2M^{0.66}(T_b - 12.7°C)}{10^3 M^{0.667}} \frac{\text{cal}}{(\text{cm}^2/\text{min}/°C)}$, from Smith (1975), and area from Benedict (1932). Warming metabolic rate is one-fourth as large.

‖ C = cooling conductance $= 0.061M^{-0.34}$ cal/cm/min/°C, from Robertson and Smith (1979).

¶ $T_b - T_w = \Delta T = \frac{\text{metabolism}}{\text{conductance}}$.

Interestingly, the head temperature was below the stomach temperature at the end of the warming experiment. This is similar to results reported by Pough and McFarland (1976) and may likewise be attributed to evaporation rather than nervous system control.

Table 2 summarizes trends of alligator thermal characteristics as functions of animal size. It provides some insights into reptilian thermoregulation in general. For example, it clearly demonstrates that endogenous heat is insignificant for small animals, but highly significant for animals over 100 kg. Obviously, activity or digestion of food could increase $T_b - T_w$. It also shows the scaling of thermal time constants and skin thickness for comparison with other reptiles. Because so many data are available over such a range in alligator size, it is tempting to extrapolate to larger animals. For example, while alligators having a mass of 1 metric ton are uncommon, marine crocodiles of 2 metric tons exist. If the thermal characteristics of crocodiles scale as they do for alligators, metabolic heat alone could increase body temperature 6.9°C in 35° water, thus requiring an increase in thermal conductance to avoid overheating. And finally a 10 metric ton dinosaur having similar thermal characteristics might have a nearly 10°C metabolic contribution while in 30°C water. Although these last comments are speculative, they illustrate the utility of having a variety of data available over a wide range of animal size.

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BOOK REVIEWS

Insect Ultrastructure Vol. 1—edited by Robert C. King and Hiromu Akai. 485 pp. 1982. Plenum Press, New York and London. $55.

This is the first of two volumes devoted to Insect Ultrastructure. It is the result of the 16th International Congress Entomology Meeting held in Kyoto in 1980. However, the chapters have been brought up-to-date. The contributors have presented detailed reviews of their subject, placing it both in a historical background and giving the up-to-date accounts of recent research.

The chapters and authors are as follows:

1. The Formation of Clones of Interconnected Cells during Gametogenesis in Insects, by Robert C. King, Joseph D. Cassidy, O.P. and Andre Rousset.
2. Relationships between Germ and Somatic Cells in the Testes of Locusts and Moths, by Annette Szollosi.
3. The Meiotic Prophase in *Bombyx mori*, by Søren Wilken Rasmussen and Preben Bach Holm.
4. Morphological Manifestations of Ribosomal DNA Amplification during Insect Oogenesis, by M. Donald Cave.
5. The Cell Biology of Vitellogenic Follicles in Hyalophora and Rhodnius, by William H. Telfer, Erwin Huebner and D. Spencer Smith.
6. Order and Defects in the Silkworm Chorion. A Biological Analogue of a Cholesteric Liquid Crystal, by Grace Dane Mazur, Jerome C. Regier and Fotis C. Kafatos.
7. The Cytoplasmic Architecture of the Insect Egg Cell, by Dieter Zissler and Klaus Sander.
8. Morphological Analysis of Transcription in Insect Embryos, by Victoria Foe, Hugh Forrest, Linda Wilkinson and Charles Laird.
9. The Morphogenesis of Imaginal Discs of Drosophila, by Dianne K. Fristrom and Wayne L. Rickoll.
10. Fine Structure of the Cuticle of Insects and Other Arthropods, by Barry K. Filshie.
11. The Structure and Development of Insect Connective Tissues, by Doreen E. Ashhurst.
12. The Structure and Development of the Tracheal System, by Charles Noirot and Cecile Noirot-Timothee.
13. Structural and Functional Analysis of Balbiani Ring Genes in the Salivary Glands of *Chironomus tentans*, by Bertil Daneholt.
14. Insect Intercellular Junctions. Their Structure and Development, by Nancy J. Lane.
15. Selectivity in Junctional Coupling between Cells of Insect Tissues, by Stanley Caveney and Robert Berdan.

The volume is well produced on glossy paper so that the EM pictures come out very well. There is a detailed bibliography to each chapter with full titles of papers. This volume is a most welcome contribution to the subject and will be of interest to all research workers on the morphology and ultrastructure of cells and tissues.

Insect Neurohormones—by Marie Raabe. 352 pp. 1982. Plenum Press, New York and London. $42.50.

This volume produces an excellent summary of the role of neurohormones in insects. The chapters are entitled: 1. Synthesis, Storage and Release of Neurohormones, 2. Control of Endocrine Gland Activity, 3. Diapause, 4. Reproduction, 5. Functioning of the Visceral Muscles, 6. Morphological and Physiological Color Change, 7. Behavior and Rhythmic Phenomena, 8. Osmoregulation, 9. Metabolism and 10. Neurohormone and Cuticle.

The author has made many contributions to the role that neurohormones play in insects, and has presented in this monograph the very helpful summary of a vast subject. There are some excellent diagrams, one of which is shown here, which summarizes the neurohormonal and glandular regulation in insects. This is compared with another diagram in the book for the system in the vertebrates. It is especially pleasing to see that the insect systems are now being considered for the role they play in the reactions of the whole animal, and that an integrative approach is emphasized in this important volume. (See Fig. 1.)

Ecology of Bats—edited by Thomas H. Kunz. 425 pp. 1982. Plenum Press, New York and London.

This volume contains chapters by 12 different authors. They discuss the roosting ecology, bat reproduction, growth and survival, the physiological ecology and energy budgets, bat activity rhythms, morphological adaptations, echolocation, insect hearing and feeding, foraging strategies of plant-visiting bats, coevolution between bats and plants, and the ecology of insect ectoparasitic on bats.

This volume presents the physiological ecology of bats in considerable detail and will indicate to the experimental physiologists many of the problems that the bats have successfully solved. Bats pollinate and disperse at least 130 plant genera. Their role as flying mammals, capable of drastic temperature reduction, efficient hunters of insects, capable of delayed fertilization, is well known. This volume presents new information on a very distinctive group of mammals.

The Hunger for Salt: An Anthropological, Physiological and Medical Analysis—by Derek Denton. 650 pp. 1982. Springer, Berlin.

This book is a wide ranging adventure and survey of the subject of the role of salt (sodium chloride) in man and animals. Most land animals and plants live some distance away from the sea and therefore have difficulty in obtaining sufficient salt for the maintenance of their intracellular and extracellular ionic balance. The sodium content of rain water decreases rapidly with distance from the ocean, so that 150 km or more from the shore, rain may be virtually salt-free. Many animal species including man show behavioural, metabolic and endocrinological evidence of sodium deficiency in the wild state. Primitive human communities may have a sodium status marginally above deficiency. Many ruminants sequester a large volume of sodium-rich salivary secretion. The animals can draw on these sodium reserves as a result of aldosterone hypersecretion by the adrenal glands. This lowers the Na/K ratio of the large salivary flow. This is proposed as one factor in the development of the success of the ruminants.

In the evolution of hominoids over 30 million years, the early prosimians were mainly vegetarian, and hunting and meat eating may have developed in the late Pliocene. Evidence from meat eating in chimpanzees indicates that over the course of a year, meat could provide an additional sodium intake of about 1 mmol/day. Denton also discusses

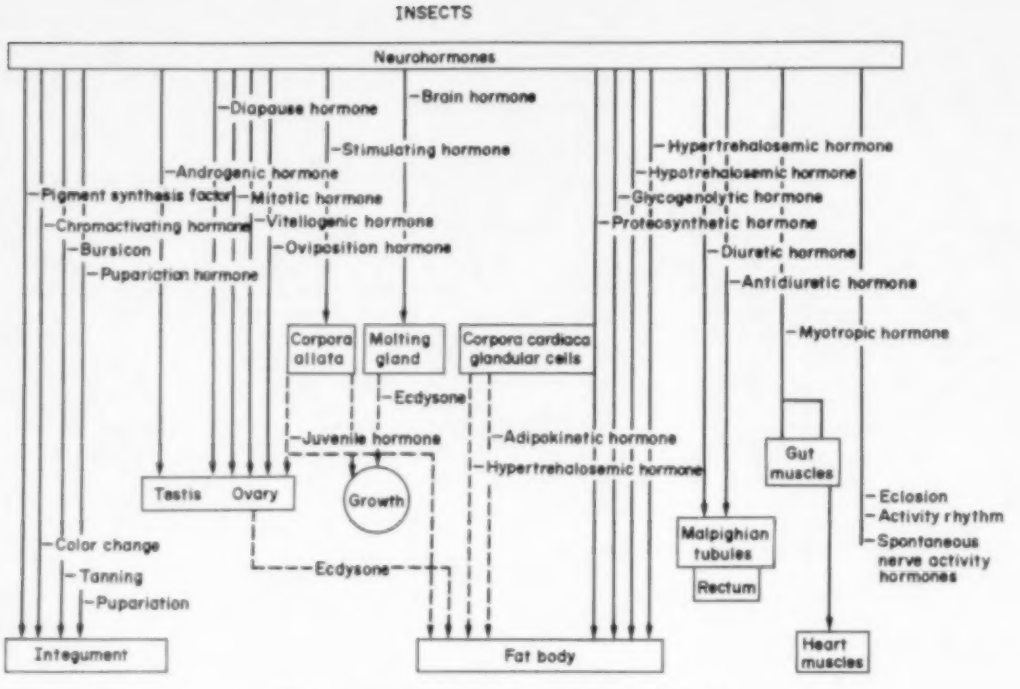

Fig. 1. From *Insect Neurohormones*, by Marie Raabe.

the role that cannibalism could play in the diet of early man. The body of a 70 kg man eaten by 10–20 people could provide the latter with sufficient calcium, phosphorus and sodium for one month. There is an excellent survey of cannibalism and the role that it could play in mineral scarce regions of the world.

A sheep that is depleted of 500–700 mmol sodium when presented with a 300 mmol/l. sodium barcarbonate solution, will rapidly drink in 2–5 min an amount closely related to the extent of the body deficit. The book discusses in detail the literature concerning sodium deficiency, salt appetite, salt receptors, the hormonal control of salt and water, the role of the renin–angiotensin system in salt control and the role of salt in hypertension. In Japan, those on a high rice diet normally take a large quantity of salt in with the rice (4–55 g/day). There is an indication of a correlation between the high salt intake and hypertension.

The author discusses the literature on every aspect of salt in a well balanced way, which besides giving interesting information also provides an exciting narrative of the key role that salt has played in the life of man and animals. It ranges from the use of the salt tax in human societies to raise money on a vital human requirement, through to the physiological, ecological and behavioural activities of animals in their struggle to obtain this essential substance.

The book should be consulted by all readers of this journal. The book provides new insights into a very important subject, and presents in a masterly way a mosaic of factual information that forms an enthralling and vivid picture of the subject.

Iron Porphyrins, Vols I and II—edited by A. B. P. Lever and Harry B. Gray. 1982. Vol. I—286 pp. £29.20. Vol. II—154 pp. £28.45. Addison Wesley, London.

These two volumes are part of a physical bioinorganic chemistry series. The common theme of this series is "what can be learnt about the geometric structures, the electronic

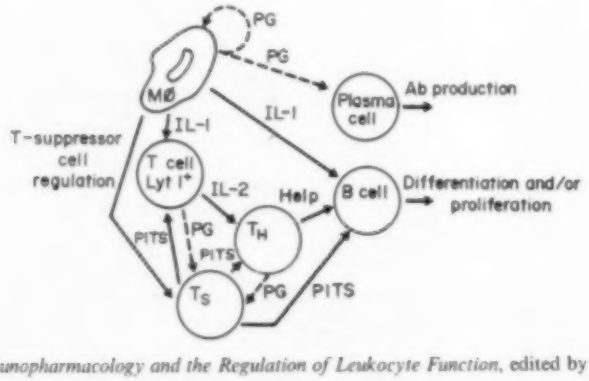

Fig. 2. From *Immunopharmacology and the Regulation of Leukocyte Function*, edited by David R. Webb.

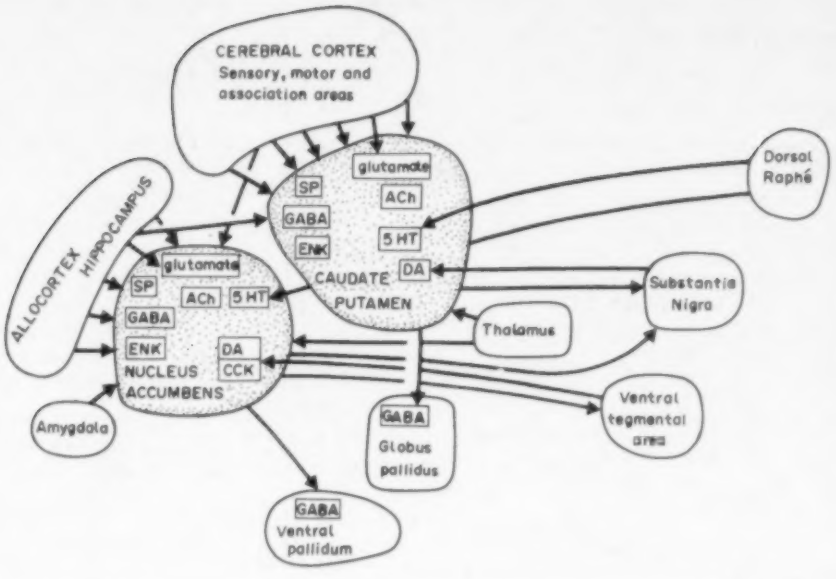

Fig. 3. From *Neurotransmitter Interaction and Compartmentation*, edited by H. F. Bradford.

structures and the mechanisms of biological redox centres through the use of modern physical techniques?"

Vol. I describes: Theoretical Investigations of Iron Porphyrins (by Gilda H. Lowe); Ligands, Spin State, and Geometry in Hemes and Related Metalloporphyrins (by Robert Scheidt and Martin Gouterman); Structural and Analytical Aspects of the Electronic Spectra of Hemeproteins (by Marvin W. Makinen and Antonie K. Churg) and Nuclear-magnetic Resonance of Iron Porphyrins (by Harold M. Goff).

Volume II covers the following subjects: Magnetic Susceptibility of Iron Porphyrins (by Samaresh Mitra); Electron Paramagnetic Resonance by Hemoproteins (by Graham Palmer); The Resonance Raman Spectroscopy of Metalloporphyrins and Heme Proteins (by Thomas G. Spiro) and the Electrochemistry of Iron Porphyrins in Nonaqueous Media (by Karl M. Kadish).

The volumes provide a very useful summary at an advanced undergraduate level and will be helpful to chemists, biochemists and medical research workers interested in understanding the chemical basis of the role that iron plays in the porphyrins. Further volumes are in preparation and will be welcomed by research workers in this important topic.

Hemoglobin and Oxygen Binding—edited by Chien Ho. 486 pp. 1982. Macmillan, London. £50.00.

Electron Transport and Oxygen Utilization—edited by Chien Ho. 446 pp. 1982. Macmillan, London. £50.00.

These two volumes are the Proceedings of an International Symposium on the Interaction between Iron and Proteins in Oxygen and Electron Transport, held in Virginia 1980. There were seven main sessions: (1) Heme–oxygen bonding; (2) Origins of co-operative oxygen binding by hemoglobin; (3) Dynamics of oxygen binding; (4) Electron transport by cytochromes; (5) Study of oxygen utilizing proteins; (6) Biochemistry of oxygen utilization and (7) Electron transport by iron sulphur proteins. Sections 1, 2 and 3 are in the first volume, whilst sections 4, 5, 6 and 7 are in the second volume.

The symposium was attended by 250 scientists and the majority of the key figures in the subject presented papers. Each session was followed by a discussion. The standard of the papers and the discussions is extremely high and the readers will find a very polished account of the state of the art and in particular, several chapters that will interest them. There is also an account of a presentation to honour Max Perutz where the "secret" background to Hb crystallography and structure is revealed. The two volumes are very well printed and illustrated and stand out from the normal run of symposia. Highly recommended.

Dietary Fiber in Health and Disease— edited by George V. Vahouny and David Kritchevsky. 330 pp. 1982. Plenum Press, New York and London. $37.50.

The importance of diet containing a higher proportion of plant cell walls, lignin, and polysaccharide has been of considerable interest to nutritionalists over the past 10 years. A high fiber diet can decrease the transit time through the gut; increase the incorporation of nitrogen into bacterial protein; increase the production of small chain fatty acids and gas in the colon; increase the loss of fecal nitrogen and lipid, decrease calcium, magnesium and iron uptake; affect the absorption of lipids and bile salts, and probably reduce the incidence of colon cancer. This volume of studies by 40 authors is a very readable (and not too bulky) account of the subject and is readily absorbed by the reader.

Presystemic Drug Elimination—edited by Charles F. George and David G. Shand. Guest editor Andrew G. Renwick. 213 pp. 1982. Butterworths, London. $39.95.

This is the first volume of a series in Butterworths International Medical Reviews, in Clinical Pharmacology and Therapeutics.

The volume is devoted to the metabolism of drugs by the liver, gastrointestinal tract, lung, and is particularly concerned with the effect of these organs on drugs taken by mouth. Some drugs are metabolized by the liver and may produce metabolites in a more toxic form than the parent drug. This is the reason that those drugs are given by injection and are not taken orally. The book is divided into four sections dealing with the major metabolic sites of drugs that are taken by mouth.

Amygdalin cyanogenic glycoside is converted to cyanide by the gut flora and is much less toxic if intramuscularly injected than if given orally. The bio-availability of aspirin is 65–75% due to its hydrolysis by the flora in the gut. Similarly the bio-availability of paracetamol, phenacetin, morphine, nalorphine, isoprenaline, leva dopa, steroids, pivampicillin, flurazepam, lignocaine, propanolol and para-aminobenzoic acid are all reduced from the theoretical availability due to metabolic action of the gut flora and other metabolic sites, such as the liver and lungs.

With more interest being shown in the pharmacodynamics of drugs, the role of the intestinal flora in metabolizing drugs and in preventing their availability is becoming more recognized. This volume, the result of a Satellite Meeting of the first World Conference of Clinical Pharmacology, is a very useful summary of the information, from experiments on animals and from clinical application in man, of the first-pass-metabolism of the gastrointestinal tract, liver and lungs.

Immunopharmacology and the Regulation of Leukocyte Function—edited by David R. Webb. 300 pp. 1982. Marcel Dekker, New York. $45.

This is Vol. 19 in the Dekker Immunology series. The use of drugs or natural products to modify the function of immunocompetent cells has led to the science of neuropharmacology, and immune modulating drugs have helped to analyse the mechanisms that control the immune response. This volume with 26 contributors summarizes different approaches to this subject. Various chemicals, such as prostaglandins, thromboxanes, hydroxy-fatty acids, lectins and bacterial lipopolysaccharides, can alter the reactions of leucocytes, macrophages and lymphocytes. This is also described in relation to the role of glucocorticoids in stress and the immune response. The subject clearly has great importance both from the practical and the theoretical view, and it is interesting to see the way in which the various chemical factors can modify the chain reactions of the immune response. The systems is very complicated as shown by the illustration of the model of PG regulation of the immunocompetent cell function taken from the paper by Webb, Wieder and Nowowiejski in this volume. (See Fig. 2.)

Neurotransmitter Interaction and Compartmentation—edited by H. F. Bradford. 839 pp. 1982. Plenum Press, New York and London. $95.

This is a volume produced as a result of a NATO advanced study meeting held in 1981. The main sections of the volume are: I. Nerve terminal organization, II. Key factors in neurotransmitter synthesis and release, III. Neurotransmitters and their interactions at the cellular level, IV. Transmitter metabolism, V. Isolated cells and tissue compartments, VI. Neurotransmitters in sub-systems of the brain and VII. Neurotransmitters in the retina.

Although, like many symposium meetings, the authors do prepare and present a set piece of work, each article is a very helpful review of the synaptic transmitter systems interactions at specific sites. Most of the regions of the mammalian brain are discussed where the information is available, and the role of the cyclic nucleotides and peptides is given considerable discussion as well as the standard transmitters, (see the figure taken from the paper by de Belleroche). The volume provides an up-to-date account of the subject and all neurophysiologists will find something of interest in it. (See Fig. 3.)

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THE PASSAGE OF ORALLY FED PROTEINS FROM MOTHER TO FOETUS IN THE RAT

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Abstract—1. Pregnant rats were orally fed with a mixture of bovine IgG, bovine albumin, ovalbumin and β-lactoglobulin. Using immunoprecipitation methods, these proteins were detected in the maternal blood serum, urine and uterine fluids, and also in the foetal blood serum and the amniotic fluid.

2. The results imply that a variety of dietary proteins, still immunoprecipitable, are able to cross the materno-foetal barriers to be detected in the foetal circulation, where they may influence the maturation of the immune system of the foetus.

INTRODUCTION

Transmission of IgG from the bloodcirculation of the mother to the foetus occurs in many species, including the rat (Brambell, 1970). This is a route by which the foetus acquires passive immunity providing an immediate protection to common antigens, e.g. infectious agents, in the extrauterine environment before its own immune system is developed. In the rat, even orally fed ^{125}I-labelled IgG and its breakdown products are transferred from the gut of the mother to her foetuses (Hemmings and Hemmings, 1979).

This work is an attempt to investigate whether proteins other than IgG, when orally fed to the mother, can cross the tissue barriers between the mother's gut and the foetus and reach the circulation of the foetus. The proteins chosen for feeding were common dietary proteins having different properties, i.e. molecular weight, and known to give rise to food allergy in man (Bleumink and Young, 1968). To detect and quantify the transmission of these proteins, immunoprecipitation methods were used. These methods may give a better picture of the uptake of proteins than the methods using radio-labelled tracers commonly in use since the latter methods can result in an overestimation of the protein uptake (Udall *et al.*, 1981).

MATERIALS AND METHODS

Nineteen female rats of the Sprague–Dawley strain (Anticimex, Stockholm, Sweden) aged about 3 months and bred at our laboratories under standard conditions, were employed in the study. Fifteen of them were pregnant between days 10 and 22, and four remained virgins. The gestational age was estimated by measuring the crown–rump length of the foetuses (Angulo, 1932).

The rats had free access to food and water until the experimental feeding, which was performed at 9 a.m. The animals were fed bovine serum, which contained 55 mg/ml of bovine IgG (BIgG) and 66 mg/ml of bovine serum albumin (BSA), mixed with the proteins chicken ovalbumin (OvA) (Sigma, St Louis, MO, USA) and cow milk β-lactoglobulin (β-LG) (Sigma) to a concentration of 30 mg/ml for both of these proteins. Each rat received 3.33 ml of this protein solution per 100 g body weight. The rats were fed under light ether anaesthesia by means of a stomach tube.

Four hours after the protein feeding, the rats were anaesthetized (Mebumal, 40 mg/kg body weight) and laparotomy was performed. Blood serum was obtained from the adults by heart puncture and from the foetuses (pooled within the litter) by bleeding after decapitation. The contents in the small intestine of the adult were obtained by flushing the proximal 20 cm with 2 ml of ice-cold 0.9% NaCl, and the supernatant was collected after centrifugation at 3000 *g* for 15 min. Adult livers were homogenized in 1 ml ice-cold 0.9% NaCl per g tissue in a Teflon–glass homogenizer. The homogenates were centrifuged at 3000 *g* for 15 min and the supernatant was saved for analysis. Samples of uterine and amniotic fluids, and urine were taken when possible.

All samples were stored frozen (−20°C) until analysis.

Detection and quantitation of the proteins were performed by electroimmunoassay (Laurell, 1966) for BSA, OvA and β-LG, and by single radial immunodiffusion (Fahey and McKelvey, 1965) for BIgG. Specific antisera for BIgG and BSA were obtained from Miles Laboratories, Freehold, USA and antisera for OvA and β-LG were raised in rabbits as described elsewhere (Jakobsson *et al.*, 1982).

RESULTS AND DISCUSSION

The results of this investigation show that food proteins, still immunoprecipitable, have the ability to cross the intestinal membranes into the blood of the rat, both pregnant and non-pregnant, and even to reach the circulation of the foetus.

In the gastrointestinal tract of the adult rats, the degradation and elimination of the fed proteins must be pronounced, since only small amounts of immunoprecipitable proteins, representing less than 1‰ of the dose given, could be detected within four hours after feeding (Table 1). Somewhat higher amounts of ovalbumin than the other proteins were found in most intestinal samples, whereas BSA and β-LG occurred only in a few intestinal samples in low amounts. The IgG molecules appeared fragmentated in the intestines, as evaluated from the multiple rings found in the single radial immunodiffusion. These results indicated that there were differences in the availability for absorption of the various protein molecules in the gut. One reason for this could be differences in resistance of the different proteins to proteolytic degradation.

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Table 1. Mean concentration ± SD in μg/ml (calculated for the samples with detectable amounts of protein) for the proteins bovine IgG (BIgG), bovine serum albumin (BSA), ovalabumin (OvA) and β-lactoglobulin (β-LG) in intestinal samples, blood serum, urine, uterine and amniotic fluids from adult and foetal rats

	Intestine	Bloodserum	Urine	Uterine fluid	Amniotic fluid
Adults, non-pregnant					
BIgG	DP (4/4)	+ (3/4)	+ (1/4)		
BSA	5.0 (1/4)	7.1 ± 3.9 (4/4)	12.0 ± 8.3 (3/4)		
OvA	38.2 ± 16.6 (4/4)	11.5 ± 11.7 (4/4)	— (0/4)		
β-LG	— (0/4)	12.7 ± 6.7 (2/4)	— (0/4)		
Adults, pregnant					
BIgG	DP (14/15)	+ (11/15)	+ (5/15)	+ (1/6)	
BSA	12.1 ± 8.6 (4/15)	9.2 ± 4.1 (12/15)	4.5 ± 3.5 (9/15)	17.2 ± 1.1 (2/6)	
OvA	34.1 ± 19.6 (11/15)	9.6 ± 5.4 (13/15)	— (0/15)	11.2 ± 3.8 (3/6)	
β-LG	12.0 ± 3.5 (2/15)	13.4 ± 7.5 (10/15)	11.3 ± 6.5 (4/15)	— (0/6)	
Foetuses					
BIgG		+ (3/15)			+ (2/7)
BSA		9.5 ± 9.2 (6/15)			30.0 (1/7)
OvA		5.7 ± 4.9 (6/15)			13.7 ± 10.2 (2/7)
β-LG		— (0/15)			— (0/7)

Figures in brackets indicate the ratio between samples with detectable amounts of the protein and the total number of samples analysed.
DP, degradation products.
+, trace amounts (< 4 μg/ml).
—, no detectable amounts.

In the blood sera of both pregnant and non-pregnant rats, the proteins fed could be detected and quantified, showing the ability of food proteins to cross the intestinal membranes (Table 1). The results showed no apparent differences in transmission between pregnant and non-pregnant rats. The amounts of the fed proteins in the sera varied considerably, both between different proteins and between individual rats (0–29 μg/ml). However, in one and the same rat the various proteins were absorbed in either relatively high or in relatively low amounts. These differences in protein transmission could be explained by differences in the intestinal proteolytic activity and/or endocytotic efficiency of the gut cells, or differences in elimination rates. In addition, food intake before the start of the experiment could affect the time of passage through the intestines, and thus the availability for absorption.

The differences observed in the uptake of the various proteins did not appear to be due to differences in the molecular weights of the respective proteins (BIgG MW : 150.000, BSA MW : 67.000, OvA MW : 43.000, β-LG MW : 18.500).

In a few liver samples, small amounts of immunoprecipitable proteins appeared. BSA was found in one liver sample at a concentration of 9.5 μg/ml, and OvA in another at a concentration of 15 μg/ml. These observations, all obtained on liver supernatants, did not agree with the high values reported by Hemmings and Hemmings (1979), who observed that the liver homogenates had the greatest uptake of ^{125}I-IgG fed to rats. The method of detection used by these investigators can also detect radio-labelled breakdown products of the fed protein whereas in our study only intact protein and relatively large, still immunoprecipitable protein products have been measured.

In the foetal blood sera, detectable amounts of BIgG, BSA and OvA were found in almost half of the samples (Tables 1 and 2). There was no major difference between the transmitted amounts of BIgG and the other proteins (BSA and OvA). This is remarkable since a selectivity for IgG via receptor-mediated uptake would have been expected (Kraehenbuhl *et al.*, 1979). Another interesting observation is that the concentration of BSA and OvA in foetal serum sometimes exceeded the concentration found in maternal serum (Table 2). These observations indicated that there was an accumulation of the absorbed proteins in the rat foetus. In addition, there was an increased concentration of the proteins in foetal sera from late gestation.

As found in this study, the rat foetus can develop in the presence of immunogenic dietary proteins derived from the mother. It is interesting to speculate about the roles of food proteins in the maturation of the immune system of mammals. One effect may be an induction of tolerance *in utero* to common food stuffs, which could

Table 2. Concentration in μg/ml of bovine serum albumin (BSA) and ovalbumin (OvA) in paired maternal and foetal blood sera (pool of the litter)

Rat No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Day of gestation	10	10	10	11	15	16	16	17	18	19	21	22	22	22	22
BSA															
Maternal bloodsera	12	10	8	8	4	—	6	20	11	8	10	8	—	8	+
Foetal bloodsera	—	—	—	—	—	—	—	2	21	2	21	—	2	9	—
OvA															
Maternal bloodsera	12	10	10	10	1	12	6	15	8	7	8	24	—	5	—
Foetal bloodsera	—	—	—	—	—	—	—	3	—	3	11	1	3	13	—

—, no detectable amounts.
+, trace amounts (< 2 μg/ml).

be of advantage to the animal. However, in special cases, particular foreign proteins could induce intolerance, resulting in a sensitization *in utero* (cf. Jarett, 1977).

High levels of BSA and OvA were also observed in some of the amniotic and uterine fluid samples. These samples came from the late gestational stages, indicating a possible transfer route via the uterine and amniotic fluids. After the foetus swallowed the amniotic fluid, the proteins studied may have entered the circulation of the foetus via the gut. In fact, it has been shown for the rat and other mammals, that proteins experimentally deposited in the amniotic fluid enter the foetal circulation via the gut (Leissring and Anderson, 1961; Lev and Orlic, 1973; Orlic and Lev, 1973). However, in our study there was no indication of higher serum levels of BSA and OvA in foetuses surrounded by amniotic and uterine fluids containing high amounts of these proteins. Studies are now in progress to evaluate further the transfer routes of proteins from mother to foetus in the rat.

It has been shown in man that food proteins, including the proteins used in this study, are transmitted to the milk in lactating mothers, resulting in symptoms appearing in their breast-fed infants (Jakobsson and Lindberg, 1983). A similar transfer of proteins as seen in the rat appears to be probable between the pregnant woman and her foetus. For understanding the biological significance of this protein transmission in man the rat may be a useful animal model.

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A COMPARATIVE STUDY ON THE STEROIDOGENIC RESPONSE DUE TO TRAINING AND ACTH TREATMENT IN THE ADRENAL CORTEX OF THE MALE RAT

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Abstract—1. The aim of this study was to ascertain the effects of training and ACTH administration on the steroidogenic *in vitro* response in the adrenal cortex of the rat when the tissue was incubated with ACTH.

2. ACTH *in vivo* treatment resulted in a highly significant increase in the steroidogenic response ($P < 0.001$) whereas training as such caused only a slight but insignificant increase in the steroidogenic responsiveness ($P > 0.05$). Training furthermore strongly suppressed the ACTH *in vivo* induced response ($P < 0.001$).

3. ACTH as such revealed the smallest effect on adrenal mass but the biggest effect on the steroidogenic response.

4. It would seem that long term exercise resulted in an overall increase in the mass and size of the adrenal glands by either increasing the size of existing cells or by increasing the number of cells or both.

The latter exercise-induced proliferation of adrenal tissue may involve an adaptive mechanism whereby larger total quantities of adrenal tissue of lowered steroidogenic efficiencies (on a mass basis) are produced in order to meet the stress resulting from the training program.

INTRODUCTION

The levels of adrenocorticotropic hormone (ACTH) in the circulation are known to increase under different conditions of stress (Allen *et al.*, 1975; Fraioli *et al.*, 1980; Collu *et al.*, 1979). The addition of ACTH *in vitro* to an incubation medium containing adrenocortical tissue, resulted in an observed, mild increase in steroid output (Zuccarello *et al.*, 1974; Goddard *et al.*, 1977). In immature rats a reduction of adrenal cholesterol was observed during the first 3 hr following intraperitoneal injection of bovine ACTH with a gradual increase to normal levels within the next 24 hr (Sayers *et al.*, 1944). No measurements on steroid output were made in the latter study, however. It seems likely that cholesterol, a common metabolite in the steroid biosynthetic pathway, is depleted with a subsequent increase in steroid hormone production in the adrenal cortex.

It was shown that the time course for the ACTH response of the adrenal cortex of humans and rats were identical (Pearlmutter *et al.*, 1974). This would lend support to possible extrapolations from results obtained with rats to humans in this field of research.

Table 1. Treadmill training program of increasing intensity (5 days/wk)

Week	Speed (m/min)	Time (min)	Total distance (m)
1	22	30	660
2	22	80	1760
3	22	130	2860
4	22	180	3960
5	27	230	6210
6	27	280	7560

The main aim of the present study was to ascertain the effects of exercise and ACTH *in vivo* injections on the steroidogenic response in adrenal cortical tissue of male rats when incubated with ACTH *in vitro*. To this end an ACTH log dose–response curve for steroid production was first obtained, which allowed the selection of appropriate ACTH concentrations for the study of steroidogenesis *in vitro*.

MATERIALS AND METHODS

Treatment of animals

Thirty-two male Long Evans rats (*Rattus norvegicus*) of the same age were obtained from the Department of Zoology, University of Port Elizabeth. The rats were assigned to either an exercising or a control (sedentary) group, consisting of 16 rats each. The exercise group was subjected to a program of treadmill running (see Table 1) until they were sacrificed. Eight of the exercised rats were also injected subcutaneously with ACTH gel (long acting ACTHAR gel supplied by Berk Pharmaceuticals S. A. (Pty) Ltd, Isando), (1 IU every 12 hr) on five consecutive days. The final injection was given on the night before they were sacrificed.

Eight of the 16 control (sedentary) rats were also injected with the same dose of ACTH for 5 days prior to sacrifice.

A treatment group of eight rats was sacrificed on the same day with 2 days between sacrificing of different treatment groups.

The exercise program was of progressive intensity over a period of 6 wk. After the sixth week the rats were exercised for another week on this final workload. The rats were sacrificed at an age of approx. 12 wk. The training program is summarized in Table 1.

In vitro *assay for steroidogenesis in the adrenal cortex*

The method of Saffran and Schally (1955), as modified by Naudé (1980), was used for the *in vitro* assay of steroidogenesis in response to ACTH in adrenal cortex slices.

On the day of sacrifice, the eight rats in a treatment group were anesthetized with pentobarbital sodium, 6 mg/100 g body mass, injected intraperitoneally. The two adrenals of each rat were removed, carefully cleaned and quartered. The eight adrenals of each rat were distributed at random in eight previously weighed weighing boats. Each weighing boat therefore contained eight adrenal quarters representing one quarter from each rat. The total adrenal mass in each weighing boat was recorded. To prevent drying out of the adrenals, the contents of each weighing boat were transferred to a Gilson respirometer flask (without side arms) containing 2 ml of Krebs Ringer Bicarbonate Glucose Buffer (KRBG) according to Umbreit *et al.* (1972). Each flask was gassed for 15 sec with 5% CO_2–95% O_2 and was then stoppered. The flasks were preincubated with continuous shaking (100–120 s.p.m.) for 1 hr at 38°C in a constant temperature waterbath. After 1 hr as much as possible of the incubation mixture was removed with the aid of a Pasteur pipette and 1.8 ml fresh KRBG buffer was added to each flask.

To four of the eight flasks, representing a particular treatment group, 100 μl of a predetermined amount of standard ostrich ACTH (13.50 μg ACTH/100 mg adrenal in 0.9% NaCl which is also 0.01 M with respect to acetic acid) was added. The remaining four flasks served as endogenous controls and each received 100 μl of the solvent medium (0.9% NaCl in 0.01 M acetic acid). Each flask was gassed for 30 sec with 5% CO_2–95% O_2 and thereafter stoppered and incubated with continuous shaking for 2 hr at 38°C.

After 2 hr 1.6 ml of the medium in each flask was immediately transferred to a cold glass stoppered tube containing 1.7 ml of cold methylene chloride (−20°C) (purified as described by Saffran and Schally (1955)). The tubes were stoppered and shaken by hand for 30 sec and were centrifuged at 1000 *g* for 5 min to separate the two phases. The organic phase was removed and care was taken that no water drops were transferred. The absorbance of the extracted steroids from each tube was read at 240 and 255 nm on a Beckman Model 35 spectrophotometer.

The steroidogenic response per mg adrenal tissue for each extract was calculated as follows:

$$\frac{\text{O.D.}_{240} - \text{O.D.}_{255}}{\text{mg Adrenal}} \times 10^3.$$

The responses of the four control flasks were averaged and the value so obtained was used to correct the response of each of the four flasks containing ACTH for endogenous steroid production. The corrected responses were averaged to give the response by a specific treatment group when incubated with ACTH *in vitro*.

Determination of optimal ACTH in vitro *dose level*

A wide range of ACTH concentrations was used to study the log dose–response curve for steroid production according to the procedure outlined above. For this preliminary study, eight rats of the same age were used. At the incubation stage of the steroidogenic assay, one flask was used as an endogenous control and the remaining seven flasks were treated with different concentrations of ACTH in 0.9% NaCl which was also 0.01 M with respect to acetic acid. The concentrations of ACTH investigated in the different incubation mixtures were 0.1, 0.5, 1, 5, 10, 50 and 100 μg ACTH/100 μl respectively. The log dose–response curve is presented in Fig. 1.

A second study was undertaken to confirm the optimal dose range of ACTH as revealed by the first preliminary study. For this purpose another group of eight rats of the same age was used. Two incubation flasks for each of three concentrations (1, 5 and 10 μg ACTH/100 μl) were used, with the remaining two flasks serving as endogenous controls. The results from this second study are also presented in Fig. 1 and confirmed the data from the preliminary investigation.

On the basis of these preliminary studies it was decided to use consistently a concentration of 13.5 μg ACTH/100 mg adrenal in all subsequent investigations. From Fig. 1 it can be seen that the selected dose was close to the midway point in the dose–response curve and hence would allow maximal sensitivity in the test system to reveal possible differences in responsiveness amongst treatment groups.

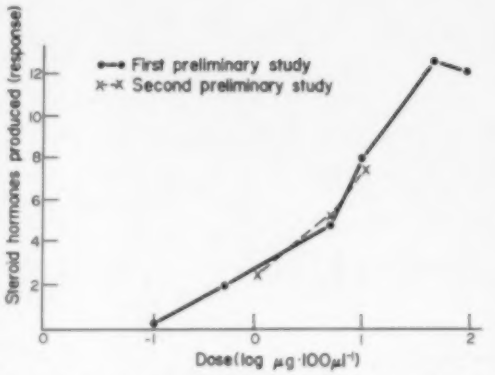

Fig. 1. Log dose–response curve for steroid production using the modified method of Saffran and Schally (1955).

RESULTS AND DISCUSSION

Body mass

The average body mass of the experimental animals in each of the four treatment groups are presented in Table 2.

From Table 2 it is evident that treatment with ACTH *in vivo* caused a significant decrease in the body mass of both control and trained animals (control + ACTH *in vivo* vs control, $P < 0.05$; trained + ACTH *in vivo* vs trained, $P < 0.02$). Training as such also resulted in a significant decrease in the body mass of the experimental animals (trained vs control, $P < 0.01$; trained + ACTH *in vivo* vs control + ACTH *in vivo*, $P < 0.02$). The latter finding is in agreement with the results of Terblanche *et al.* (1981).

In vitro *steroidogenesis in the adrenal cortex*

The results are presented in Table 3. All recorded responses have been corrected for endogenous steroid production as described under Materials and Methods.

Based on the average adrenal and body masses of each treatment group (Table 2), the percentage of adrenal mass to body mass for each treatment group was calculated (Table 3).

Table 2. Average body mass (g) of rats on the day of sacrifice

Treatment groups			
Control	Control + ACTH *in vivo*	Trained	Trained + ACTH *in vivo*
393.79 ± 15.15	337.18 ± 15.92*	325.42 ± 7.81†	286.18 ± 8.18‡§

Values are means ± SEM (average for eight rats).
*Control + ACTH *in vivo* vs control, $P < 0.05$.
†Trained vs control, $P < 0.01$.
‡Trained + ACTH *in vivo* vs trained, $P < 0.02$.
§Trained + ACTH *in vivo* vs control + ACTH *in vivo*, $P < 0.02$.

From the results presented in Table 3 it is clear that training as such causes only a slight but insignificant increase in the *in vitro* steroidogenic response of the rat adrenal ($P > 0.05$). However, treatment with ACTH *in vivo* produced a highly significant stimulation of the subsequent *in vitro* steroidogenic response as compared to the value obtained with the control group ($P < 0.001$).

The most interesting feature is the highly significant difference between the results obtained from the two ACTH *in vivo* treated groups. When ACTH was injected into trained rats, the training had a suppressing effect on the subsequent steroidogenic response. Thus training by itself may produce a small stimulating effect, but when combined with ACTH injections, the effect was significantly lower than that observed with the ACTH *in vivo* treatment alone ($P < 0.001$). However, the combination of training + ACTH *in vivo* still produced a significantly increased *in vitro* steroidogenic response over that of the control group ($P < 0.05$).

From Table 3 it is also evident that both ACTH *in vivo* and training separately and in combination resulted in apparent increases in the mass of the adrenal glands in comparison with the control animals. This is especially apparent in the trained group where an increase of 28.3% in average adrenal mass was observed. This finding is in agreement with the results of Groenewald *et al.* (1978). In the case of training + ACTH *in vivo* the increase was 17.4% and in the case of control + ACTH *in vivo* the increase was 4.5%. On visible examination the above mentioned treatments also caused an increase in the size of the adrenals. There seems to be an inverse relationship between the percentage increase in steroidogenic response and the percentage increase in adrenal mass.

In the trained group the highest percentage increase in adrenal mass was coupled with the lowest percentage increase in steroidogenic response and *vice versa* for the control + ACTH *in vivo* treatment group.

When ACTH was injected into trained rats the increase in adrenal mass was suppressed and the steroidogenic response was slightly elevated relative to the trained group as such. ACTH as such revealed the smallest effect on adrenal mass but showed the biggest effect on the steroidogenic response.

A final interpretation of the present data concerning trained as opposed to ACTH treated animals is not possible at the present stage and would have to include a consideration of the relative effects of these treatments on specific properties of the adrenal receptors concerned, including possible changes in affinity and total receptor numbers per cell. The present data would seem to imply that the two treatments have antagonistic overall effects insofar as ACTH treatment strongly elevates the overall steroidogenic responsiveness per unit mass of adrenal tissue whereas this effect is largely counteracted by long term training which instead appears to stimulate a strong apparent increase in adrenal mass. The data therefore could possibly indicate the proliferation of larger quantities of adrenal tissue of relatively lower steroidogenic efficiencies (on a mass basis) as compared to ACTH *in vivo* treatment. This overall effect could result from either lowered numbers of receptors per cell or a lowered receptor affinity for ACTH in the trained animals. The apparent increase in total adrenal mass would then be seen as a compensatory mechanism. Further elucidation of the mechanism(s) involved in adrenal adaptation to exercise and training will have to await detailed studies at the receptor level.

From Table 2 it can be seen that the different treatments had a significant effect on the body masses with the control rats exhibiting the highest mass followed in decreasing order by the following treatments: control + ACTH *in vivo*, training and training + ACTH *in vivo*. The adrenal masses on the other hand tend to increase in the opposite order with the result that the ratio adrenal mass : body mass also strongly increases in the opposite direction of body mass for the four groups concerned (Table 3). Unfortunately the adrenal mass data did not allow statistical analysis in the present study, seeing that the recorded masses refer to randomized samples derived from the different animals in each group. The data nevertheless does seem to support rather clearly the tendencies discussed above.

In summary, ACTH *in vivo* treatment as well as training as such caused a significant decrease in the body masses of the experimental animals subjected to these treatments relative to the appropriate control groups. ACTH *in vivo* treatment resulted in a highly significant increase of the *in vitro* steroidogenic response whereas training as such caused only a slight but insignificant increase in the latter response. When ACTH was injected into trained rats, training revealed

Table 3. Adrenal steroid response after *in vitro* treatment with 13.5 μg ostrich ACTH/100 mg adrenal mass. Each value represents eight rats

Variable	Treatment groups			
	Control	Control + ACTH *in vivo*	Trained	Trained + ACTH *in vivo*
Response	3.36 ± 0.51	8.76 ± 0.22*†‡	4.41 ± 0.30	5.16 ± 0.38§
% Response increase relative to control	0	160.7%	31.2%	53.6%
Average adrenal mass (mg)	49.78	52.01	63.88	58.43
% Increase in adrenal mass	0	4.5%	28.3%	17.4%
$\frac{\text{Adrenal mass}}{\text{Body mass}} \times 100$	0.01264%	0.01543%	0.01963%	0.02037%

Response values are means ± SEM.
*Control + ACTH *in vivo* vs Control, $P < 0.001$.
†Control + ACTH *in vivo* vs Trained, $P < 0.001$.
‡Control + ACTH *in vivo* vs Trained + ACTH *in vivo*, $P < 0.001$.
§Trained + ACTH *in vivo* vs Control, $P < 0.05$.

a suppressing effect on the steroidogenic response relative to the control + ACTH *in vivo* treatment groups. In the trained-injected rats the increase in adrenal mass as a result of training was suppressed but the ratio of adrenal mass relative to the body mass was slightly increased and the response slightly higher relative to the trained group (no ACTH *in vivo*). ACTH treatment as such revealed the smallest effect on adrenal mass but the biggest effect on response.

It can possibly be concluded that *in vivo* treatment with ACTH stimulates the ACTH-receptors resulting in an increased steroid hormone response. Therefore an increase in the levels of ACTH in the circulatory system possibly results in an increase in the activity of the ACTH-receptors on the adrenal cortex cell membranes.

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STUDIES OF THE EFFECT OF EXPERIMENTAL INFLAMMATION ON RAT LIVER NUCLEOTIDE SUGAR POOLS

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Abstract—1. The effect of experimental inflammation on levels of nucleotide sugars was studied in rat liver.

2. There was an increase of about 2-fold in the levels of UDP-*N*-acetylhexosamines and GDP-Man at 8 and 4 hr after inflammation, respectively. At 48 hr after inflammation GDP-Man had returned close to control values, but UDP-*N*-acetylhexosamines were still about 50% above controls.

3. There was a 30% reduction in CMP-NeuAc and UDP-Gal at 8–12 hr after inflammation before increasing to slightly above controls at 16–48 hr after inflammation.

4. Inflammation resulted in an increase in activities of glucosamine-6-phosphate synthase and UDP-GlcNAc-2′-epimerase to about twice control activities at 24 and 8 hr after inflammation, respectively, before declining; CMP-NeuAc synthase activities did not show large changes following inflammation.

INTRODUCTION

Inflammation in mammals caused by a variety of conditions including neoplastic disease, rheumatoid arthritis and exposure to chemical inflammatory agents results in changes in a variety of processes affecting lipid, carbohydrate, amino acid and mineral metabolism (Biesel, 1980). Recently, we have reported that inflammation causes an increase in amino acid pools in liver (Woloski *et al.*, 1983). This was attributed to an increase in the flux of amino acids from muscle to plasma and then to liver for utilization by liver for elevated protein synthesis. The major group of liver synthesized proteins that are elevated in inflammation are the acute phase serum glycoproteins, such as rat α_1-acid glycoprotein which has been extensively studied in our laboratory (e.g. Jamieson *et al.*, 1975, 1982; Shutler *et al.*, 1977; Langstaff *et al.*, 1980). Acute phase glycoproteins, like α_1-acid glycoprotein, contain carbohydrate chains of the complex type composed of Man, Gal, GlcNAc and NeuAc (Jamieson, 1983). The activated forms of these sugars that are utilized for oligosaccharide chain synthesis are the appropriate nucleotide sugars. In view of the effect of inflammation on hepatic synthesis of acute phase glycoproteins, as well as the earlier reported changes in hepatic amino acid pools found following inflammation, we have now measured the pools of nucleotide sugars in liver following inflammation and compared these results with those obtained from control animals. The results are discussed in terms of the possible relationship to elevated glycoprotein biosynthesis found following inflammation and should prove to be useful in understanding the complex metabolic changes occurring in liver during injury and stress.

Abbreviations: GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine; Gal, galactose; Man, mannose; Glc, glucose; NeuAc, *N*-acetylneuraminic acid; UDP-GlcNAc, uridine diphosphate-*N*-acetylglucosamine; UDP-GalNAc, uridine diphosphate *N*-acetylgalactosamine; GDP-man, guanosine diphosphate mannose; CMP-NeuAc, cytidine monophosphate-*N*-acetylneuraminic acid.

MATERIALS AND METHODS

Sugars and nucleotide sugars were from Sigma Chemical Corp., St. Louis, MO; UDP-[^{14}C] GlcNAc (300 nCi/nmol); GDP-[^{14}C]Man (166 nCi/nmol) and CMP-[^{14}C] NeuAc (197 nCi/nmol) were from New England Nuclear Corp., Lachine, Quebec; ACS cocktail was from Amersham Corp., Oakville, Ontario; analytical grade Dowex 1-x8 100-200 mesh, Cl^- form was from Bio-Rad Laboratories, Richmond, CA; picric acid was from BDH, Poole, U.K.

Preparation of liver extracts

Liver extracts were prepared from control and experimentally inflammed rats as described earlier for the measurement of liver amino acid pools (Woloski *et al.*, 1983) except that [^{14}C] labelled nucleotide sugars were added (typically 0.1 nmol, 30 nCi) prior to deproteinization by homogenization with picric acid; this was necessary to test for recoveries following chromatography on Dowex columns. Samples of 8 ml supernatants were applied to Dowex columns as before (Woloski *et al.*, 1983) and nucleotide sugars were eluted with 40 ml 0.25 N HCl after amino acids, sugars and amino sugars had been washed off the columns with 40 ml 0.04 N HCl (Woloski *et al.*, 1983). Based on recoveries of radioactivity not less than 90% of the nucleotide sugars in the liver homogenates were eluted in the 0.25 N HCl fraction. The 0.25 N HCl fractions were taken to dryness by rotary evaporation and used for analyses of UDP-*N*-acetylhexosamines, UDP-Gal and GDP-Man. For analyses of CMP-NeuAc, cell sap fractions were prepared from 4 g samples of liver by a procedure based on that described by Bley *et al.* (1973). Livers were perfused with ice-cold 0.154 M KCl–1 mM EDTA adjusted to pH 7.5 with NaOH and homogenized in perfusion buffer using 10 up and down strokes of a Potter–Elvehjem homogenizer at 2000 rev/min. A large granule fraction was sedimented at 20,000 *g* for 10 min in the Sorvall RC-2B with the SS-34 rotor and the supernatant centrifuged for 1 hr at 105,000 *g* in a Beckman L5-50 fitted with a Ti 50 rotor. The supernatant was used for determination of CMP-NeuAc (see

below) and for assay of CMP-NeuAc synthase and UDP-GlcNAc-2′-epimerase (see below). For assay of glucosamine-6-phosphase synthase activities (see below) homogenates were fractionated as above, but 12 mM glucose-6-phosphate was added prior to homogenization to prevent conversion of fructose-6-phosphate to glucose-6-phosphate by phosphoglucose isomerase (Miyagi and Tsiuki, 1971; Bley *et al.*, 1973).

Determination of nucleotide sugars

The 0.25 HCl effluents from the Dowex columns (see above) were dissolved in 2 ml water; 1.5 ml were removed for analysis of UDP-*N*-acetylhexosamines and the remainder was used for analysis of GDP-Man and UDP-Gal.

For analysis of UDP-GlcNAc and UDP-GalNAc the sample was adjusted to 2 N HCl by addition of 1.5 ml 4 N HCl and hydrolysed at 100°C for 3 hr under vacuum to release free amino sugars (Molnar *et al.*, 1964); samples were taken to dryness in a desiccator over NaOH. Samples were dissolved in 1 ml water and appropriate volumes analyzed in an NC-2P Technicon Amino Acid Analyzer System. To test for recovery after hydrolysis, samples of 100 nmol UDP-GlcNAc, UDP-GalNAc, glucosamine–HCl and galactosamine–HCl, either alone or as mixtures, were analyzed as above. Recoveries were not less than 90%.

For analyses of GDP-Man and UDP-Gal, 0.5 ml 0.02 N HCl and 100 nmol myo-inositol were added to the solution that remained after removal of a sample for hexosamine analyses (see above). Nucleotide sugars were hydrolyzed at 100°C for 20 min to release free sugars. The samples were applied directly to columns of Dowex-1 × 8 as described previously (Woloski *et al.*, 1983) and neutral sugars eluted with 40 ml 0.04 N HCl; the effluent was taken almost to dryness by rotary evaporation, transferred to 1 ml Reactivials (Pierce Chemical Co., Rockford, IL) dried under N_2 at 65°C and used for analyses of sugars by gas–liquid chromatography. Gas–liquid chromatography was performed on alditol acetate derivatives of sugars prepared by the method of Torello *et al.* (1980). The equipment used was a Perkin-Elmer Sigma 2B gas chromatograph fitted with a flame ionization detector. Glass columns 6 ft long and 2 mm i.d. were packed with 3% SP-2330 on 100/120 Supelcoport (Supelco Inc., Bellefonte, PA). The temperature program was set for 6 min at 180°C followed by 2 min at 220°C at a rate of 1°C/min and finally for 15 min at 250°C at a rate of 5°C/min. Injection port temperature was 220°C and the detector temperature was 300°C. The carrier gas was N_2 at a rate of 20 ml/min. Peak areas were determined with a Perkin-Elmer M-2 integrator.

Analyses for CMP-NeuAc was performed on cell fractions using a method based on that described by van den Eijnden and van Dijk (1972). Samples of 38 nCi CMP-[^{14}C]-NeuAc (197 nCi/nmol) were added to 1 ml vol of cell sap in ice and the entire solution applied to a 40 cm origin on Whatman 3 MM paper (46 × 57 cm) and descending chromatography performed at 4°C for 24 hr using 95% ethanol : 0.6 M NH_4OH (7 : 3) as solvent. The band containing CMP-NeuAc was detected under u.v. light or by counting small sections of paper cut from the edges of the chromatogram. Under the conditions used CMP-NeuAc had an R_f value of about 0.3 and was well separated from NeuAc which had an R_f value of about 0.5. The CMP-NeuAc was eluted from the chromatogram with water and the eluate freeze-dried. The CMP-NeuAc typically contained 90% of the radioactivity applied to the chromatogram. The freeze-dried material was dissolved in 1 ml water and suitable volumes were removed for counting and for determination of NeuAc using the thiobarbituric acid method of Warren (1959); the method includes a hydrolysis step which releases NeuAc from CMP-NeuAc *in situ*. The CMP-NeuAc content of cell sap was calculated from the results of quantitative analysis for NeuAc and the radioactivity recovered, after correcting for NeuAc added as CMP-[^{14}C]-NeuAc.

In all the experiments involving recovery of sugars, results were corrected for losses based on the recoveries of radioactivity obtained following the isolation procedures or, in the case of gas–liquid chromatography, by using myo-inositol as an internal standard.

Enzyme assays

Glucosamine-6-phosphate synthase activities were measured according to Kornfeld (1967). Assay mixtures contained 12 mM glutamine, 1 mM EDTA, 40 mM sodium phosphate buffer, pH 7.5, and up to 0.1 ml 105,000 *g* supernatant containing 12 mM glucose-6-phosphate (see above) and 6 mM fructose-6-phosphate in a total volume of 0.5 ml. Incubations were for 60 min at 37°C and the reaction was stopped by heating at 100°C for 2 min. Protein was removed by centrifugation at 5000 *g* for 10 min in a bench centrifuge and 0.2 ml samples of supernatants were analyzed for glucosamine-6-phosphate by a modification of the Morgan–Elson method as described by Ghosh *et al.* (1960) scaled down to a final volume of 3.6 ml. One unit of glucosamine-6-phosphate synthase activity is defined as 1 nmol glucosamine-6-phosphate formed in 60 min.

UDP-GlcNAc-2′-epimerase was assayed according to Sommar and Ellis (1972). Assay mixtures contained 0.5 μmol UDP-GlcNAc, 12.5 μmol $MgSO_4$, 50 μmol Tris–HCl buffer, pH 7.5, and up to 0.1 ml 105,000 *g* supernatant in a total volume of 0.25 ml. Samples were incubated for 20 min at 37°C and the reaction was stopped by heating at 100°C for 2 min and *N*-acetyl-D-mannosamine, the reaction product, was determined as described by Spivak and Roseman (1966). One unit of UDP-GlcNAc-2′-epimerase activity is defined as 1 nmol *N*-acetyl-D-mannosamine formed in 20 min.

For assay of CMP-NeuAc synthase the procedure of Kean and Roseman (1966) was used. Each incubation mixture contained 90 μmol Tris–HCl, pH 9.0, 2.5 μmol NeuAc, 2.5 μmol $MgCl_2$ and 0.1 ml of 105,000 *g* supernatant in a total volume of 0.5 ml. Incubations were for up to 20 min at 37°C and the tubes were cooled in ice. Free NeuAc was destroyed with 0.075 ml 2.5 M $NaBH_4$ for 15 min at room temperature and CMP-NeuAc remaining was assayed as above (Warren, 1959). One unit of CMP-NeuAc synthase activity is defined as 1 nmol CMP-NeuAc formed in 20 min.

In all enzyme assays linearity was established between enzyme activities and amount of enzyme protein; reactions were also linear with time under the assay conditions used. Protein was assayed as described by Lowry *et al.* (1951), but with modified reagents and volumes described by Miller (1959).

RESULTS

The effect of experimental inflammation on hepatic levels of UDP-*N*-acetylhexosamines is shown in Fig. 1. Control rat liver contained 141 nmol of UDP-*N*-acetylhexosamines/g wet wt liver composed of 95 nmol UDP-GlcNAc and 46 nmol of UDP-GalNAc. Inflammation caused a rapid rise in UDP-*N*-acetylhexosamine pools reaching a maximum at 8 hr after inflammation where the pool sizes were about twice those found in controls. This was followed by a rapid decline reaching a value of about 50% above controls at 12 hr after inflammation; a second minor peak was observed at 16 hr after inflammation before a steady state was established where UDP-*N*-acetylhexosamine pools were about 50% above control values (Fig. 1). Figure 1 also shows that the response of UDP-GlcNAc to inflammation parallels that of UDP-GalNAc although the pool of UDP-GlcNAc was about twice that of UDP-GalNAc. This observation was not surprizing since it is known that UDP-GlcNAc-4′-epimerase which forms UDP-GalNAc from UDP-GlcNAc maintains steady state

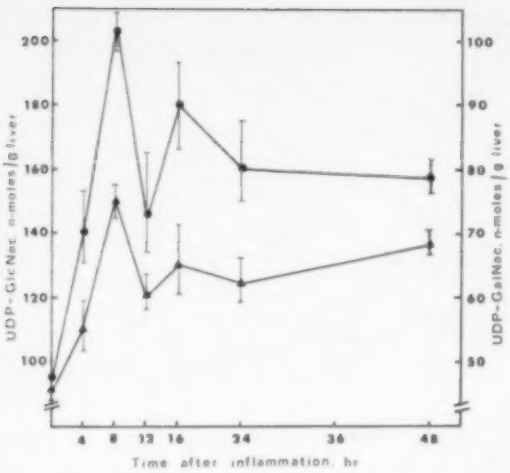

Fig. 1. Effect of inflammation on UDP-GlcNAc, —●—; and UDP-GalNAc, —▲—; in liver. Results are means from 3 to 6 experiments; the bars represent the range of values obtained expressed as standard errors of the mean.

levels of these nucleotide sugars such that UDP-GlcNAc levels are about twice those of UDP-GalNAc (Molnar *et al.*, 1964).

Figure 2 shows that the control level of GDP-Man was 4.8 nmol/g wet wt liver. Inflammation resulted in a rapid increase to about 70% above controls within 4 hr after inflammation; there was then a rapid decline to about half control values at 12 hr followed by a second peak at 24 hr before returning close to control values at 48 hr after inflammation. Although the levels of GDP-Man were much lower than those found for UDP-*N*-acetylhexosamines (Fig. 1) the response of GDP-Man to inflammation did bear a resemblance to that found with UDP-*N*-acetylhexosamines. For example, all three nucleotide sugars increased rapidly to about twice control values at short times after inflammation (i.e. at 4 hr for GDP-Man and 8 hr for UDP-*N*-acetylhexosamines; Figs 1 and 2), declined at 12 hr after inflammation and then increased again at longer times after inflammation (see Figs 1 and 2).

The effect of inflammation on hepatic levels of CMP-NeuAc and UDP-Gal are shown in Fig. 3. The control level of CMP-NeuAc was 49 nmol/g wet wt liver, but this was reduced by about 30% at 8–12 hr after inflammation; there was then a rapid increase to give values slightly above control values at 24–48 hr after inflammation. The response of UDP-Gal to inflammation seemed to parallel that of CMP-NeuAc (Fig. 3). The control level of UDP-Gal was 31 nmol/g wet wt liver. Like CMP-NeuAc the level of UDP-Gal declined at short times after inflammation before increasing rapidly to just above control values at 16–48 hr after inflammation (Fig. 3).

In view of the alterations described above in the hepatic levels of UDP-*N*-acetylhexosamines and CMP-NeuAc found during inflammation, it was of interest to measure the changes in the activities of the regulatory enzymes involved in the synthesis of these two nucleotide sugars. Glucosamine-6-phosphate synthase which converts fructose-6-phosphate to glucosamine-6-phosphate with glutamine as amide donor is the regulatory enzyme for UDP-GlcNAc synthesis; the enzyme is subject to feedback inhibition by UDP-GlcNAc (Kornfeld *et al.*, 1964; Schachter, 1978). For synthesis of CMP-NeuAc the regulatory enzyme is UDP-GlcNAc-2′-epimerase which catalyzes the formation of *N*-acetylmannosamine from UDP-GlcNAc; this enzyme is subject to feedback inhibition by CMP-NeuAc (Kornfeld *et al.*, 1964; Schachter, 1978). CMP-NeuAc synthase was also measured as an example of a non regulatory enzyme. The effect of inflammation on these three enzyme activities is given in Table 1. Both regulatory enzymes showed increases in specific activities following inflammation. There was a small increase in glucosamine-6-phosphate synthase activity at 8 hr after inflammation, but the major response occurred at 24 hr after inflammation when enzyme activities were about twice those of controls. Although this was followed by a decline in activities, values were still substantially above control values at 48 hr after inflammation. Activities of UDP-GlcNAc-2′-epimerase declined to about 60% of controls at 4 hr after inflammation (Table 1); however, this was followed by a rapid rise to over twice control values at 8 hr after inflammation after which activities returned to control values (Table 1). In contrast to the two regulatory enzymes, activities of CMP-NeuAc synthase did not change significantly following inflammation (Table 1).

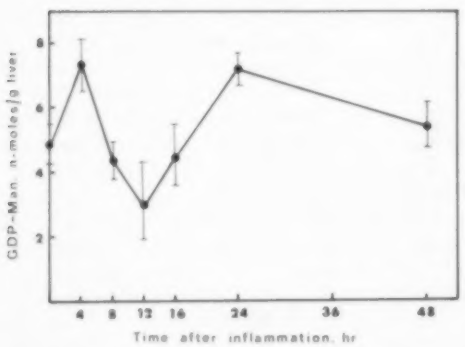

Fig. 2. Effect of inflammation on GDP-Man in liver. Results are expressed as for Fig. 1.

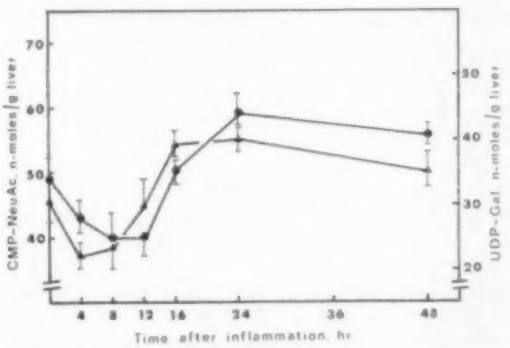

Fig. 3. Effect of inflammation on CMP-NeuAc, —●—; and UDP-Gal, —▲—; in liver. Results are expressed as for Fig. 1.

Table 1. Effect of inflammation on activities of glucosamine-6-phosphate synthase, UDP-GlcNAc-2'-epimerase and CMP-NeuAc synthase*

Time after inflammation (hr)	Glucosamine-6-P synthase	UDP-GlcNAc-2'-epimerase	CMP-NeuAc synthase
Control	35.0 ± 2.5	58.6 ± 1.9	90.0 ± 4.6
4	38.5 ± 2.9	41.0 ± 2.3	86.5 ± 4.5
8	42.5 ± 1.5	127.7 ± 2.0	85.0 ± 1.4
12	37.0 ± 0.8	96.7 ± 1.9	75.0 ± 3.4
16	59.5 ± 5.8	70.2 ± 2.6	71.0 ± 3.5
24	78.0 ± 4.4	60.9 ± 1.2	101.0 ± 7.7
48	60.0 ± 2.1	56.8 ± 1.2	95.0 ± 3.5

*Expressed as specific enzyme activities (units of enzyme activity/mg cell sap protein, see Materials and Methods section). Values are means from six experiments on separate animals ± SEM.

DISCUSSION

The value of 141 nmol of UDP-*N*-acetylhexosamines found for control liver compares favourably with some literature values, but is slightly lower than others. For example, Okubo *et al.* (1976) reported values ranging from 127 to 534 nmol/g liver from fed rats; with starved rats, Bley *et al.* (1973), reported 207 nmol/g liver and Bates *et al.* (1966) reported 270 nmol/g liver. The variability in UDP-*N*-acetylhexosamine levels could either reflect the use of different strains of rat, or result from variations in the nutritional status of the animals; UDP-GlcNAc pools have recently been shown to be affected by diet (Tepperman *et al.*, 1981). Increases in UDP-*N*-acetylhexosamine pools following trauma have been reported, but the earliest times that were examined were at 18 hr. (Bley *et al.*, 1973; Okubo *et al.*, 1976). At times after trauma of 18 hr or longer it was observed that UDP-*N*-acetylhexosamine pools increased by about 20–30% which compares favourably with the 50% increase found in the present work at longer times after inflammation (Fig. 1). The earlier workers, however, would not have detected the sudden rise in UDP-*N*-acetylhexosamine pools to over twice control values that occurred at 8 hr after inflammation, or the subsequent dip in pool levels found at 12 hr after inflammation (Fig. 1). These results show that the response of UDP-*N*-acetylhexosamine pools to inflammation is much more rapid than was previously believed.

Compared to the information available on UDP-*N*-acetylhexosamine pools in liver, the literature contains much less information on liver pools of the other nucleotide sugars examined in the present studies and how the pools respond to trauma. The value of 49 nmol/g liver found for CMP-NeuAc is slightly higher than the 37 nmol/g liver reported by Carey and Hirschberg (1979), but the response of this nucleotide sugar to trauma has not been reported. It was found that CMP-NeuAc pools were reduced by about 30% at 8–12 hr after inflammation before returning to control levels at about 16 hr after inflammation. A similar response was observed for UDP-Gal pools. The control level of UDP-Gal was 31 nmol/g liver which is substantially lower than the value of 92 nmol/g liver reported by Bauer *et al.* (1976), using an enzyme assay system, rather than analysis by gas chromatography, to measure the nucleotide sugar. Although the reason for this discrepancy is unclear, Bauer *et al.* (1976) did report that UDP-Gal pools fell by 20–30% at short times after trauma induced by sham operation or partial hepatectomy and that this was followed by an increase to control levels at 24–48 hr after operation. The behaviour of UDP-Gal pools in response to trauma reported by Bauer *et al.* (1976), is thus consistent with the data presented in the present work. The control level of GDP-Man was 4.8 nmol/g liver which is considerably lower than the pool sizes of the other nucleotide sugars studied. Although the literature does not appear to contain information on liver GDP-Man levels, low levels of this nucleotide sugar have been found in other tissues. For example, Mendicino and Rao (1975) reported a value of 12 nmol/g tissue for lymph node. As mentioned in Results the behaviour of the GDP-Man pools after inflammation more closely resembled the changes observed in UDP-*N*-acetylhexosamine pools, rather than those found for CMP-NeuAc and UDP-Gal. Although the magnitude of the changes were different, pools of UDP-*N*-acetylhexosamines and GDP-Man both increased rapidly after onset of inflammation, then declined and finally increased again (Figs 1 and 2).

The similarities found for the effect of inflammation on the behaviour of the CMP-NeuAc and UDP-Gal pools on the one hand and of the UDP-*N*-acetylhexosamine and GDP-Man pools on the other, could be explained if we examine the role played by these nucleotide sugars as donors of the glycosyl units for the synthesis of the oligosaccharide chains of liver glycoproteins. Residues of NeuAc and Gal are known to occupy terminal and penultimate positions, respectively, on oligosaccharide chains of liver synthesized glycoproteins, in addition, it is well known that these two sugars are transferred directly from their nucleotide sugars by the appropriate glycosyl transferases to growing oligosaccharide chains (e.g. Schachter, 1978; Beyer *et al.*, 1981). Thus, although CMP-NeuAc synthesis is under regulatory control (see Results; also, see below), the pool levels of CMP-NeuAc and UDP-Gal might be expected to change in a similar way in response to inflammation; moreover, the changes are likely to be related to changes in glycoprotein biosynthesis found after inflammation. Therefore, it is interesting to speculate that the decline in CMP-NeuAc and UDP-Gal pools at 4–16 hr after inflammation might reflect increased demands for NeuAc and Gal for liver glycoprotein biosynthesis which is substantially increased at 8–24 hr inflammation

(Jamieson *et al.*, 1975). Incorporation of GlcNAc into oligosaccharide chains of glycoproteins occurs in two distinct ways; the first way involves direct transfer from UDP-GlcNAc to a growing oligosaccharide chain in the same way that NeuAc and Gal are added. The second way is quite different and involves the formation of a lipid–oligosaccharide complex which acts as the glycosyl donor for the sugars that form the inner core regions of the carbohydrate chains of serum glycoproteins (Sharon and Lis, 1981; Jamieson, 1983). The lipid–oligosaccharide also contains all of the Man residues found in the oligosaccharide chains of serum glycoproteins. Thus, because of the complex way in which Man and GlcNAc are incorporated into glycoproteins it is harder to relate changes in GDP-Man and UDP-GlcNAc pools in inflammation directly to changes in glycoprotein biosynthesis. However, since transfer of Man and GlcNAc to glycoproteins involves formation of intermediate lipid–oligosaccharide complexes it is not surprising that pools of GDP-Man and UDP-GlcNAc show similar changes following inflammation.

Glucosamine-6-phosphate synthase, the regulatory enzyme for UDP-GlcNAc formation has been studied extensively in past years and it has been found to be regulated in a complicated way (e.g. Kornfeld *et al.*, 1964; Bates *et al.*, 1966; Kornfeld, 1967; Miyagi and Tsiuki, 1971). Briefly, the enzyme is subject to feedback inhibition by UDP-GlcNAc, but the degree of inhibition is dependent on secondary factors that can alter the binding constant of UDP-GlcNAc to the enzyme; these factors include UTP, AMP and glucose-6-phosphate. Also, it appears that the enzyme is normally about 90% inhibited which leaves substantial latitude for regulatory control. The specific activity of the enzyme in control livers was 35 units/mg protein which is slightly lower than the 40 units/mg protein reported by Bley *et al.* (1973) and the 35.5–46 units/mg protein reported by Okubo *et al.* (1976). In the present studies glucose-6-phosphate was added to the assay system to prevent the conversion of fructose-6-phosphate to glucose-6-phosphate by phosphoglucose isomerase which is present in high levels in liver (Miyagi and Tsiuki, 1971). However, as mentioned above, glucose-6-phosphate can enhance the feedback effect of UDP-GlcNAc on the enzyme and so this probably explains the slightly lower activity of glucosamine-6-phosphate synthase found in the present work compared with the data presented by Bley *et al.* (1973), and Okubo *et al.* (1976). Inflammation resulted in a small increase in enzyme activity at 8 hr followed by a decline to control values at 12 hr after which there was a rapid rise to about twice that of control activities at 24 hr after inflammation (Table 1). Although there was a decline at 48 hr after inflammation values were still substantially above controls. These results are consistent with the findings of Bley *et al.* (1973) and Okubo *et al.* (1976) who reported large increases in glucosamine-6-phosphate synthase activities following laparotomy. The times studied by these workers were 18 hr to 8 days after injury when it was found that the glucosamine-6-phosphate synthase response could be blocked by actinomycin D or cycloheximide suggesting that the elevated activities of glucosamine-6-phosphate synthase found after 18 hr of injury are mainly due to the synthesis of new enzyme protein, rather than by regulatory control. However, it is difficult to reconcile the behaviour of the enzyme at early times after inflammation (i.e. up to 12 hr after turpentine injection) with the 2-fold increase in the pool size of UDP-GlcNAc found at 8 hr after inflammation (Fig. 1). Although there is an elevated level of glucosamine-6-phosphate synthase activity at 8 hr after inflammation the change is not large and it would be unlikely to account entirely for the increased pool of UDP-GlcNAc found at this time after inflammation. Clearly, the feedback control of glucosamine-6-phosphate synthase by UDP-GlcNAc during inflammation is a complex process; moreover, the process cannot be considered in isolation since UDP-GlcNAc also acts as the substrate for UDP-GlcNAc-2′-epimerase, the first enzyme in the pathway leading to CMP-NeuAc formation. This reaction is not detectably reversible (Sommar and Ellis, 1972) and provides a means to reduce the GlcNAc pool and channel the UDP-GlcNAc into the biosynthetic pathway leading to CMP-NeuAc. The control activity of UDP-GlcNAc-2′-epimerase was 58 units/mg protein which is comparable to the value of 44.7 units/mg protein calculated from the data reported by Kikuchi *et al.* (1971) (Kikuchi *et al.* reported 134 units/mg protein of activity defining a unit as 1 nmol product formed/hr; in the present work a unit is 1 nmol product formed/20 min). Inflammation resulted in a rapid reduction in the activity of UDP-GlcNAc-2′-epimerase to about 60% of the control value at 4 hr after inflammation. Although it is not clear what the explanation is for this response, one outcome would be a decreased utilization of UDP-GlcNAc which could partially explain the rapid rise in the UDP-GlcNAc pool after inflammation (Fig. 1). At 8 hr after inflammation epimerase activity increased to twice control values. This change might be due to the feedback inhibitor for the enzyme, CMP-NeuAc, which is present in substantially reduced levels at 8–12 hr after inflammation.

The acute phase response of glycoproteins has been studied for many years, but so far there has been no satisfactory explanation for the phenomenon. The present studies have focused on one aspect of the process, namely the behaviour of nucleotide sugar pools during inflammation, concentrating on those nucleotide sugars that are the precursors of the sugars found in the oligosaccharide chains of acute phase glycoproteins. In addition, the effect of inflammation on some enzymes of nucleotide sugar metabolism was also studied. Large changes were observed in the activities of glucosamine-6-phosphate synthase and UDP-GlcNAc-2′-epimerase, the two regulatory enzymes studied; but the non-regulatory enzyme, CMP-NeuAc synthase, did not change substantially. Although it is difficult to interpret information from *in vitro* assays of the enzymes in crude preparations, especially when they are under feedback control, it is of interest to note that both regulatory enzymes showed elevated activities during the time when liver glycoprotein biosynthesis is increasing rapidly (i.e. at 8–24 hr after inflammation, Jamieson *et al.*, 1975). In addition, it is clear that the response of the nucleotide sugar pools to inflammation is very rapid with substantial increases in GDP-man and UDP-*N*-acetylhexosamine pools being evident as early as 4 hr

after inflammation. Glycoprotein biosynthesis is just starting to show some stimulation at 4 hr after inflammation so changes in hepatic pools of GDP-Man and UDP-*N*-acetylhexosamines appear to precede elevated protein synthesis.

The results described in this paper add to our knowledge on changes previously reported (Jamieson *et al.*, 1975; Shutler *et al.*, 1977; Langstaff *et al.*, 1980; Woloski *et al.*, 1983) that accompany the acute phase response to inflammation. In addition, the results emphasize the importance of recognizing early biochemical changes in liver and suggest that short time course studies may be crucial in achieving a full understanding of the acute phase response of glycoproteins to inflammation.

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SEASONAL CHANGES IN THERMOREGULATION OF THE RACCOON DOG (*NYCTEREUTES PROCYONOIDES* GRAY 1834)

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Abstract—1. Oxygen consumption (ml × $kg^{-0.75}$/min) in relation to ambient temperature (T_a) in the raccoon dog whelps at the ages of 7–9 weeks and 17–19 weeks is described by equations $y = 32.9 - 0.69x$ and $y = 26.2 - 0.49x$, respectively. The corresponding equations to adults in summer and winter pelages are $y = 19.6 - 0.46x$ and $y = 14.5 - 0.32x$, respectively.

2. The cooling constant (min^{-1}) of deceased raccoon dogs decreased exponentially with increasing body mass, while $M^{-0.75}$ specific heat transfer coefficient (W × $kg^{-0.75}$/°C) regressed linearly on body mass, $y = 0.124 - 0.00066x$.

3. Cooling rate of deceased animals were more dependent on body mass than on pelage quality.

INTRODUCTION

Arctic mammals are often forced to live under a wide range of thermal conditions. Their young are especially sensitive to thermal changes because of the relatively greater amount of exposed surface in relation to body mass and reduced fur insulation (Blix, 1979). The typical means by which Arctic mammals adjust their energetics to changing climates involve a seasonal change in the degree of fur insulation (Hart, 1956). For instance, in Arctic fox (*Alopex lagopus*), the difference in fur insulation between summer and winter pelage lies at about 50% (Scholander *et al.*, 1950; Underwood and Reynolds, 1980).

Climate may affect the basal metabolic rate (BMR) of some mammals (Hart, 1957). The lower critical temperature (T_{lc}) also changes with season (Hart, 1957). The influences of seasonal pelage change on metabolic response to ambient temperature (T_a) have been demonstrated in several Arctic mammals (Irving *et al.*, 1955; Hart *et al.*, 1965; Underwood, 1971; Casey *et al.*, 1979; Casey and Casey, 1979).

The raccoon dog (*Nyctereutes procyonoides* Gray 1834) is a species basically adapted to a north temperate climate in Eastern Asia. It has been introduced into the European part of the USSR (Heptner *et al.*, 1974; Cajander *et al.*, 1979; Safonov, 1980). At present, its range extends north to 63°N and south to the Caucasus Mountains in the USSR (Corbet, 1978). It has also extended westwards into Eastern Europe including Finland (c.f. Notini, 1948; Nowak and Pielowski, 1964; Bajohr, 1968; Lloyd, 1977). This dog-like species has acclimatized well because of its food habits, low number of natural enemies, and thermoregulatory properties. Its ability to hibernate during the coldest winter periods has been considered to be expecially important in this regard (e.g. Korhonen *et al.*, 1983a).

In our previous paper (Korhonen *et al.*, 1983b), thermoregulation of the raccoon dog in winter pelage was studied by determining its metabolic response to different T_as. In addition, the insulative value of winter pelage and its importance for thermal conductance were clarified. Until now, however, similar data have not been available for the raccoon dog in summer pelage during the summer and autumn. The present study was conducted in order to provide those data. Furthermore, additional winter data were collected for comparison with our earlier study (Korhonen *et al.*, 1983b).

MATERIALS AND METHODS

Four groups of raccoon dogs were studied from June to December 1982. Whelps born in the spring constituted three groups and were tested at the ages of: 7–9 weeks, 15 June to 2 July (Group 1); 17–19 weeks, 22 August to 1 September (Group 2); and 7 months, 26 November to 6 December (Group 3). The first two groups were in summer pelage and were sexually immature. The animals in the third group were in winter pelage and were sexually mature. The fourth group comprised adult animals, slightly over 1 year of age, and was tested between 13 and 29 July while in summer pelage. Details on numbers, sexes and weights are as follows:

Group 1: $n = 6$ (3 males, 3 females), 1.15–1.50 kg.
Group 2: $n = 8$ (5 males, 3 females), 4.3–6.4 kg.
Group 3: $n = 5$ (1 male, 4 females), 5.1–8.6 kg.
Group 4: $n = 5$ (2 males, 3 females), 7.0–9.9 kg.

Metabolic response to T_a by raccoon dogs was measured using the open flow respirometric technique. Oxygen consumption at various T_as (range from +24 to −15°C) was determined with a Beckman OM-14 Oxygen Analyzer and recorded by a Servogens chart recorder. A metabolic chamber measuring 36 × 29 × 12 cm (length × width × height) was used for the young whelps (Group 1), and one of 53 × 36 × 33 cm for the adults and older whelps.

Dried air was pumped through the metabolic chamber at a flow rate ranging from 3400 to 4400 ml/min depending on the age of the test animals. Air temperature inside the chamber was controlled with a thermocouple and recorded with an Ellab du 3s (Copenhagen) thermometer. Before testing, raccoon dogs were caged outdoors, and water and conventional farm feed (originated from Koillis-Savon rehu Ltd., Juankoski, Eastern Finland) was supplied *ad libitum*. Animals were deprived of food for about 12 hr prior to the experiments which usually began at 0830 hr. All mea-

surements were made during normal daylight hours. The animals were allowed to adapt to the respiration chamber prior to each measurement. The value used for calculating metabolic rate was that obtained after the animal had reached a steady level of oxygen consumption and maintained it for 20–30 min. Oxygen consumption was calculated according to the method of Depocas and Hart (1957).

Freshly killed raccoon dogs were used for determination of thermal conductance. They were weighed and placed with a thermocouple inserted into their rectum on a styrofoam mat at a T_a of about -20°C. The animals were allowed to cool and their rectal temperatures were read to the nearest 0.1°C for about 4 hr. Thermal conductance was calculated by the method of Morrison and Tietz (1957) assuming a specific heat of 3.4 J/g per °C. To evaluate the importance of the guard hair in heat loss, two adult raccoon dogs without guard hair (s.c. samson fur) were killed in mid-November, and their cooling rates were determined as above.

Heat transfer coefficients (minimum thermal conductances) were determined using three methods: (1) calculating thermal conductances from the cooling rate curves of freshly killed animals (C_d), (2) fitting a curve of oxygen consumption value below thermoneutrality to T_a (C_f), and (3) calculating minimum thermal conductances from oxygen consumption measurements at T_as below thermoneutrality (C_m) (c.f. McNab, 1980; Korhonen *et al.*, 1983b).

Mean values have been expressed as $\bar{X} \pm$ SE. Comparisons between two means were made using Student's t-test.

RESULTS

Figure 1 shows the relation between oxygen consumption and T_a from Group 1 whelps (7–9 weeks old) in early summer. At thermal neutrality, resting metabolic rate (RMR) was relatively constant with a mean value of 23.1 ± 0.42 ml $O_2 \times kg^{-0.75}$/min (7.69 ± 0.08 W $\times kg^{-0.75}$/min). The lower critical temperature (T_c) was about $+15$°C. Below thermoneutrality, oxygen consumption increased linearly with decreasing T_a. This increase can be described by the equation $y = 32.9 - 0.69x$.

In the late August, RMR of older whelps was 17.7 ± 0.84 ml $O_2 \times kg^{-0.75}$/min (5.89 ± 0.27 W $\times kg^{-0.75}$/min).

The lowest ambient temperature at which they could maintain their body temperature (T_b) without an increase in metabolic rate was about $+15$°C. This was of the same order of magnitude as 10 weeks earlier. Below this T_a the elevation of oxygen consumption was expressed by the equation $y = 26.2 - 0.49x$. The slope of the curve was less than in late June (Fig. 1). When extrapolated to zero oxygen consumption, both equations intercept T_a at over $+45$°C.

Oxygen consumption vs T_a in adult raccoon dogs is shown in Fig. 2. At thermoneutrality, RMR of raccoon dogs in summer pelage was 12.4 ± 0.55 ml $O_2 \times kg^{-0.75}$/min (4.14 ± 0.17 W $\times kg^{-0.75}$/min). The T_c in summer was about $+15$°C. Below this, the rise in oxygen consumption with declining T_a is described by the equation $y = 19.6 - 0.46x$. The RMR for adults in winter pelage was 10.4 ± 0.33 ml $O_2 \times kg^{-0.75}$/min (3.49 ± 0.11 W $\times kg^{-0.75}$/min). This RMR was significantly lower ($P < 0.05$) than that of adult raccoon dogs in summer pelage. The T_c in winter was about $+11$°C which is slightly lower than that in summer. Elevation of oxygen consumption in winter below thermoneutrality was also lower than in summer. When extrapolated to zero oxygen consumption, the lines for adult raccoon dogs in summer and winter pelage intercept T_a at about the T_b ($+39$°C) of the raccoon dog.

Cooling rate and thermal conductance of deceased animals were more dependent on body mass than on pelage quality. Thus, the smallest animals cooled most rapidly (Tables 1 and 2). Cooling rate in relation to body mass was described by a decreasing

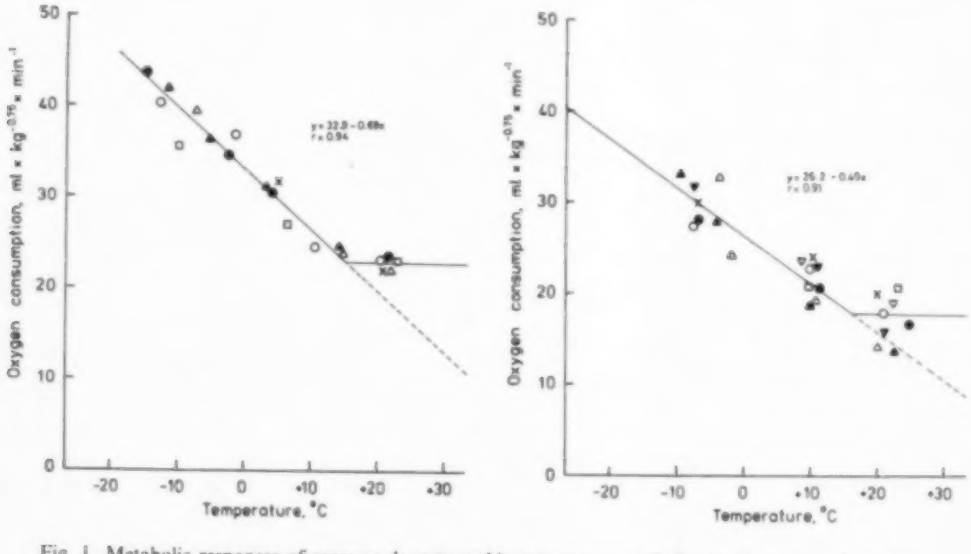

Fig. 1. Metabolic responses of raccoon dogs to ambient temperature. Left: whelps, 7–9 weeks of age. Right: whelps, 17–19 weeks of age. The curves of metabolism on temperature below thermoneutrality were fitted by means of least squares.

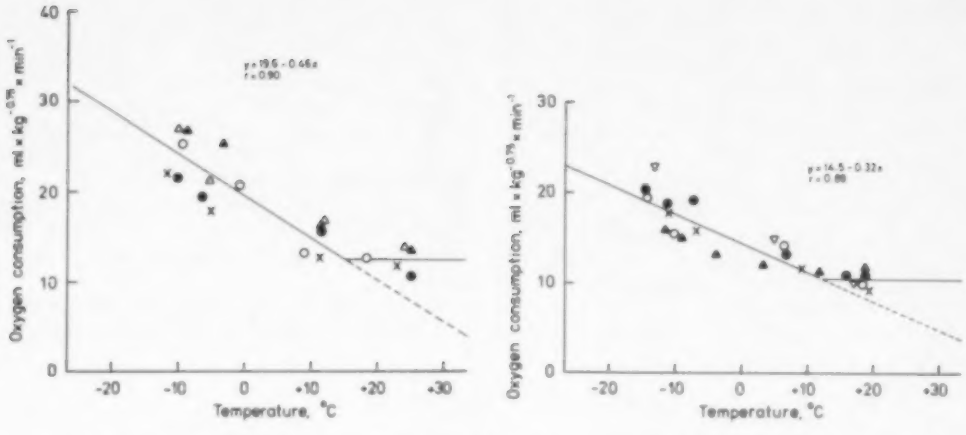

Fig. 2. Metabolic responses of raccoon dogs to ambient temperature. Left: adults in summer pelage. Right: adults in winter pelage. The curves of metabolism on temperature below thermoneutrality were fitted by means of least squares.

Table 1. Body mass, energy metabolism, cooling constant, heat transfer coefficient for cooling rate of dead animals (C_d) and for fitted (C_f) and mean conductances (C_m) of live animals, and calculated critical gradient ($\Delta T = RMR/C_m$) of animals

Sex	Mass (kg)	RMR (W × kg$^{-0.75}$)	Cooling constant (min^{-1})	Heat transfer coefficient (W × kg$^{-0.75}$/°C) C_d	C_f	C_m	C_m/C_f	C_m/C_d	ΔT (°C)
♂	1.15	7.69	—	—	0.310	0.330	1.1	—	23
♀	1.22	7.70	0.00201	0.122	0.255	0.295	1.2	2.4	26
♀	1.23	7.83	—	—	0.315	0.305	1.0	—	26
♀	1.24	7.70	—	—	0.286	0.300	1.0	—	26
♂	1.49	7.29	0.00189	0.119	0.306	0.305	1.0	2.6	24
♂	1.50	7.93	—	—	0.310	0.306	1.0	—	26
♀	1.85	—	0.00164	0.110	—	—	—	—	—
♀	1.90	—	0.00184	0.123	—	—	—	—	—
♀	4.3	6.22	0.00099	0.082	0.232	0.228	1.0	2.8	27
♀	4.4	6.35	—	—	0.244	0.255	1.1	—	25
♂	4.4	6.62	—	—	0.224	0.260	1.2	—	26
♂	4.5	6.32	—	—	0.274	0.271	1.0	—	23
♀	5.3	4.55	—	—	0.284	0.245	1.0	—	19
♂	5.7	6.68	—	—	0.245	0.256	1.1	—	26
♂	5.8	4.72	0.00102	0.091	0.215	0.232	1.1	2.5	20
♂	6.4	5.72	—	—	0.253	0.254	1.0	—	23
♂	6.6	—	0.00076	0.070	—	—	—	—	—

Conversion factor of 4.8 cal/ml O_2 is used. 1 Watt = 1.163 kcal/hr.
Upper part: whelps, 7–9 weeks of age. Lower part: whelps, 17–19 weeks of age.

Table 2. Body mass, energy metabolism, cooling constant, heat transfer coefficient for cooling rate of dead animals (C_d) and for fitted (C_f) and mean conductances (C_m) of live animals, and calculated critical gradient ($\Delta T = RMR/C_m$) of animals

Sex	Mass (kg)	RMR (W × kg$^{-0.75}$)	Cooling constant (min^{-1})	Heat transfer coefficient (W × kg$^{-0.75}$/°C) C_d	C_f	C_m	C_m/C_f	C_m/C_d	ΔT (°C)
♂	5.1	3.92	—	—	0.148	0.150	1.0	—	26
♀	6.7	4.58	0.00076	0.070	0.185	0.190	1.0	2.7	24
♀	6.8	4.52	0.00098	0.090	0.197	0.197	1.0	2.2	23
♀	6.9	4.18	—	—	0.180	0.175	1.0	—	24
♀	8.6	3.52	—	—	0.159	0.143	0.9	—	25
♂	11.4	—	0.00060	0.063	—	—	—	—	—
♀	4.4	—	0.00104*	0.086	—	—	—	—	—
♂	7.0	—	0.00080*	0.074	—	—	—	—	—
♀	7.0	3.52	—	—	0.124	0.125	1.0	—	28
♀	7.6	3.31	0.00078	0.074	0.107	0.115	1.1	1.6	29
♂	8.0	3.42	0.00072	0.069	0.154	0.150	1.0	2.1	23
♀	9.0	3.95	—	—	0.118	0.109	0.9	—	36
♂	9.9	3.24	—	—	0.129	0.121	1.0	—	27

*Samson.
Conversion factor of 4.8 cal/ml O_2 is used. 1 Watt = 1.163 kcal/hr.
Upper part: adults in summer pelage. Lower part: adults in winter pelage.

exponential curve (Fig. 3a). $M^{-0.75}$ specific heat transfer coefficient, on the other hand, decreased linearly with the increasing body mass (Fig. 3b). However, there were no seasonal differences in cooling rates ($P > 0.05$). In addition, the same cooling constants were measured for a samson raccoon dog weighing 7.0 kg and one with a "normal" pelage weighing 7.6 kg.

Heat transfer coefficients calculated from metabolic data (C_f, C_m) were clearly higher for live animals than for dead animals as determined from cooling data. C_f was about the same order as C_m.

The data obtained allowed us to calculate the critical thermal gradient (ΔT) which shows the T_{lc} below which energy metabolism must increase. The slope of the critical gradient ($T_b - T_{lc}$) is equal to M/C_m, where M = metabolic rate at a certain T_a below thermoneutrality (W × kg$^{-0.75}$/min) and C is the minimum thermal conductance at that temperature (W × kg$^{-0.75}$/min) (c.f. Scholander *et al.*, 1950). In late June the calculated ΔT for growing whelps (7–9 weeks of age) varied from 24 to 29°C, and about 2 months later it varied from 19 to 27°C. For adults, the calculated summer values ranged between 23 and 26°C and winter values between 23 and 36°C.

We also estimated the energy savings as a result of seasonal change in insulation (Table 3). Daily energy

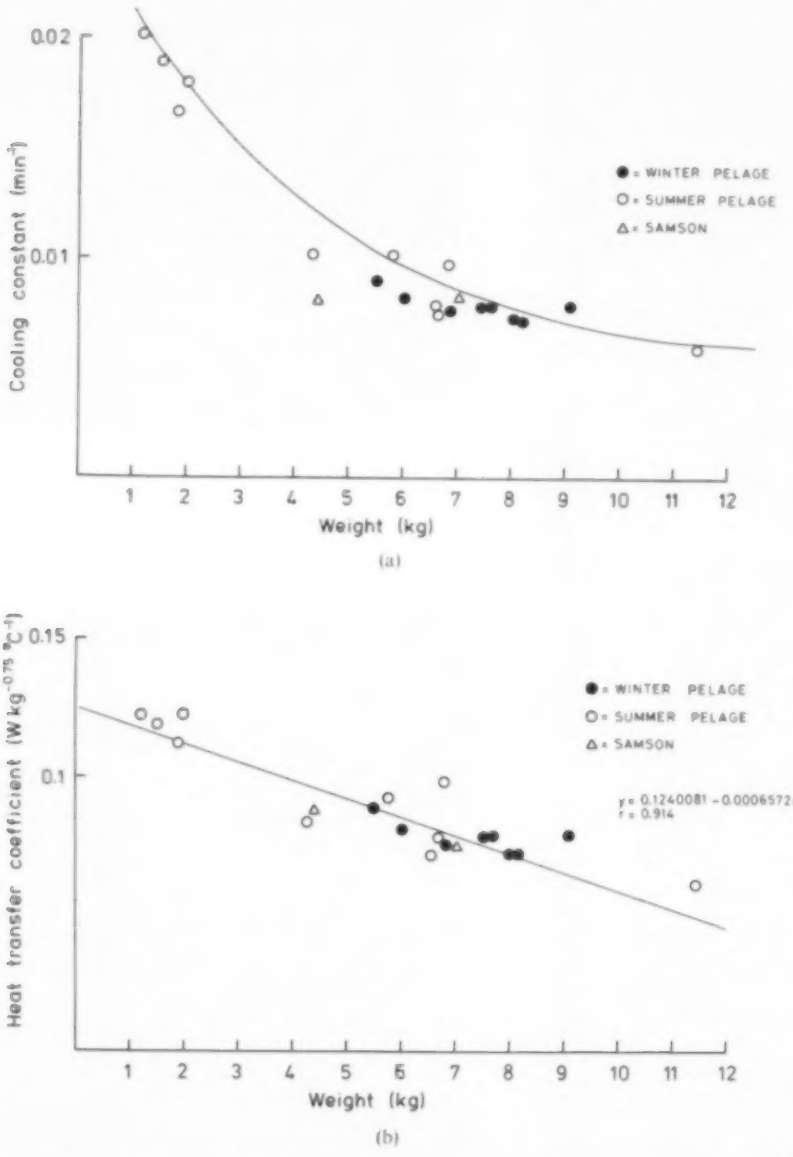

Fig. 3. (a) Cooling constants of deceased raccoon dogs as a function of body mass. The curve is fitted by eye. (Data sources: present study, Korhonen *et al.*, 1983b; Korhonen, unpublished data.) (b) $M^{-0.75}$ specific heat transfer coefficients of deceased raccoon dogs as a function of body mass. (Data sources: present study, Korhonen *et al.*, 1983b; Korhonen, unpublished data.)

savings increase when T_a decreases being about twice as great at a T_a of -30°C as that at a T_a of 0°C.

DISCUSSION

The lower RMR of the raccoon dog in winter pelage as compared to those in summer pelage suggests that there could be a seasonal shift in RMR. In our previous paper (Korhonen *et al.*, 1983b) we also measured the RMR of adult raccoon dogs in winter pelage. The RMR of those animals averaged 3.71 ± 0.24 W × kg$^{-0.75}$/min. These values, however, do not differ significantly from our present summer values ($P > 0.05$). Studies on many Arctic mammals like Arctic fox support the conclusion that in general there would be no seasonal change in basal (resting) metabolic rate (c.f. Scholander *et al.*, 1950; Irving *et al.*, 1955; Heroux, 1961; Casey *et al.*, 1979; Casey and Casey, 1979).

On the other hand, it should be noted that the behaviour of raccoon dogs differs from that of many other canids; for example they are able to spend cold winter periods hibernating in burrows or dens (e.g. Mäkelä, 1973; Heptner *et al.*, 1974). Hock (1960) called this kind of hibernation winter lethargy which implies that there is no actual decrease in T_b and metabolism. In many mammals hibernation provides significant energetic savings. Morrison (1960) stated that hibernation is only found in animals which are unable to obtain adequate external food reserves. This is, in fact, the situation for wild raccoon dogs; during the cold, snowy winter, their ability to find enough food is restricted. Therefore, they prepare themselves for hibernation by increasing stores of body fat (c.f. Heptner *et al.*, 1974). Hibernation and a concurrent shift to lower RMR are, in many species, often parallel adaptations to the seasonal shortage of food (c.f. McNab, 1974; Miller, 1978). However, although we found a significant difference between summer and winter values of RMR, magnitude of the difference was so small that it probably lacks physiological importance. On the other hand, Sokolov (1949) stated that metabolism of the raccoon dog could be lowered by about 25% during hibernation.

The T_{lc} of the adult raccoon dogs with winter pelage was about 5°C lower than that of summer ones when calculated from oxygen consumption values. Thus, there appears to be a seasonal shift in critical temperature. However, in comparison to Arctic fox with a shift of 30°C, this is rather low (c.f. Underwood, 1971). On the other hand, it should be noted that this T_{lc} is based on mathematical calculations and shows only the general trend. Hart (1957) emphasized that thermal experience of animals exposed to cold in the laboratory often is different from that in nature. Therefore, changes in basal metabolism derived from laboratory studies as an index of climatic adjustment to cold under natural conditions must be used cautiously. In addition, a question arises, "What is the real temperature experienced by test animals?", especially at lower T_as. Radiation and conduction in a small metabolic chamber could confuse the situation. Yet, wind velocities in test chambers are often lower than those in the nature, and therefore mix calculation of metabolic rates (c.f. McNab, 1980).

Conductances determined from cooling data of sacrificed raccoon dogs are the same order of magnitude whether the animals are in summer or winter pelage. Thus, we suggest that differences in cooling constants are consistent with differences in body mass. For an adult raccoon dog with a body mass of 11.4 kg and summer pelage, a minimum value for cooling constant was obtained. According to Siivonen (1974), the heaviest body weight that a raccoon dog is able to achieve lies at about 12 kg. This is supported also by our own experience. A 1 cm layer of subcutaneous fat provides thermal insulation comparable to 0.5 cm of sheeps wool (c.f. Monteith, 1973). Hence, we feel that the conductance of our animal with body mass of 11.4 kg and subcutaneous fat layer of 2–4 cm, is the minimum value that a raccoon dog can reach by body mass insulation. We also measured the amount of subcutaneous fat in a raccoon dog weighing 10.5 kg on October 30. Subcutaneous fat formed about 30% of the whole carcass weight (2.750 kg). Thus, cooling rate curves are probably reliable for the comparison of the insulative values of the pelages of different animals only if the animals are comparable in body mass and composition. The importance of guard hairs as a thermal insulation seems to be rather low as our samson animals indicated. However, our measurements were carried out in still air. Wind certainly would reduce even more the thermal protection provided by samson pelage when compared to normal pelage.

Determination and reliability of thermal conductance have been criticized in recent years (c.f. Birkebak, 1966; Tracy, 1972; Bakken and Gates, 1975; Bakken, 1976; Marsh, 1979; McNab, 1980; Hissa *et al.*, 1982). McNab (1980) stated that "it represents an unacceptably simple version of a complex interaction". Main pathways by which heat is lost from the mammalian body are conduction, convection, radiation, and evaporation of water. The heat exchange per °C difference between T_b and T_a is called thermal conductance. Thermal conductance determined from cooling rate curves of dead animals is lower than that based on metabolic data of live animals because the former is not influenced by blood circulation, respiratory heat loss and metabolism. In addition, heat loss by conduction is dependent on the surface on which the test animal is lying (c.f. Korhonen *et al.*, 1983b). On the other hand, a dead animal cannot control its posture and air circulation around its body.

The ratio C_m/C_f demonstrates the extent by which C_f generally underestimates the true minimum conductance (C_m). If $C_m/C_f = 1.0$, thermal conductances should be correctly estimated (c.f. McNab, 1980). As listed in Tables 1 and 2, the ratio of C_m/C_f in most cases lies around 1.0.

Estimated energy savings as a result of seasonal insulation change at -30°C in the raccoon dog (41%) was slightly lower than that determined in Arctic fox. Casey *et al.* (1979) calculated that for the Arctic fox about 53% of RMR was saved at that T_a.

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Table 3. Estimated energy savings as a result of seasonal insulation change in the raccoon dog

T (°C)	M (summer) ($W \times kg^{-0.75}$)	M (winter) ($W \times kg^{-0.75}$)	Difference (S-W) ($W \times kg^{-0.75}$)	(C_a) RMR saved	Daily energy saving (Kcal/day)
TNZ*	4.14	3.49	0.65	—	—
0	6.56	4.85	1.71	19	48
−10	8.03	5.96	2.07	23	58
−20	9.71	7.03	2.68	34	75
−30	11.20	8.03	3.17	41	89

Metabolism (M) is calculated from data given in Fig. 2 and Table 2.
*Thermoneutral zone.

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MAGNETIC MATERIAL IN EUROPEAN EEL (*ANGUILLA ANGUILLA* L.)

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Abstract—1. Samples of the European eel (*Anguilla anguilla* L.) were investigated for magnetic material by measurements of magnetic susceptibility, χ.

2. Bone samples from the skull, vertebral column and pectoral girdle contain magnetic precipitates with a saturation magnetization of the order of 10^{-3} emu g^{-1}.

3. In samples from the premaxilloethmovomerine block we find magnetic material in the preiosteum and/or the meninx, and the bone yields a purely diamagnetic background with $\chi_0 = -(0.45 \pm 0.02) \cdot 10^{-6}$ emu g^{-1}.

4. The location of the magnetic material in the other bones, having the same χ_0, is analogous. This was not conclusively shown due to the difficulty to separate the bones from the connecting membranes.

INTRODUCTION

Biologically synthesized magnetite was first found in the radular cappings of chitons (Lowenstam, 1962) and later in bacteria (Blakemore, 1975), where chains of single domain size magnetite crystals cause the bacteria to line up with, and become orientated along, the geomagnetic field (Frankel *et al.*, 1978). This demonstration of an orientation response to geomagnetism started a search for ferromagnetic material in several animals, with a demonstrated or suspected ability to detect magnetic fields. Magnetic material has been found in among other species honey bee (Gould *et al.*, 1978; Kuterbach *et al.*, 1982), homing pigeon (Walcott *et al.*, 1979; Presti and Pettigrew, 1980), woodmouse (Mather and Baker, 1981), Pacific dolphin (Zoeger *et al.*, 1981), and even in man (Kirschvink, 1981; Baker *et al.*, 1983).

That the ferromagnetic deposits could possibly form a basis for magnetic field perception has been argued by Yorke (1979) and by Kirschvink and Gould (1981), but so far no such magnetic sense organ has been identified and described. The ability of several animal species to detect and orientate themselves to the geomagnetic field is however well established, e.g. in homing pigeons (Keeton, 1971), honey bees (Lindauer and Martin, 1972) and Elasmobranch fish (Kalmijn, 1978). The European eel (*Anguilla anguilla* L.) performs a spectacular spawning migration, across the North Atlantic to the Sargasso Sea. It is largely unknown what orientation cues are used during the migration. Laboratory experiments give some evidence that eels could use the geomagnetic field, either indirectly by detecting the electric current field induced, when the fish or the water moves in the geomagnetic field (Rommel and McCleave, 1973), or by direct detection of the magnetic field (Branover *et al.*, 1971; Tesch, 1974).

The present study of magnetic material in eels was undertaken in connection with an investigation of eel behaviour in natural and artificially altered magnetic fields (Karlsson, in preparation). The measurements show that eels do contain magnetic material, in approximately the same concentrations as in the species discussed above. The localization is diffuse, with all dissected samples of bone showing magnetic precipitates.

MATERIALS AND METHODS

Sample preparation

The eels used in the study were in the silver eel stage, caught when migrating along the Swedish coast. After anaesthesia with Ms 222 (Sandoz) the eels were perfused via the ventral aorta with aerated Cortland saline (Wolf, 1963) containing 5000 IE/l Na-heparin (Sigma Inc., U.S.A.) to remove all blood. The perfusion was continued until the gills were white and the perfusate coming from the venous return to the heart was completely colourless.

We did not investigate the whole fish. Samples were taken from the head but also from other parts of the body, the vertebral column and the pectoral girdle. Dissection was made with nonmagnetic tweezers and tungsten or beryllium–copper scalpels. After dissection the samples were dried in vacuum. The sample sizes were in the range of about 10–30 mg dry wt. The eel has a specialized anatomy in the skull region, distinguishing it from other fishes. The upper jaw is directly connected to the cranium and since many bones are fused, bone samples from the head contain parts of more than one bone. Figure 1 shows the location of the two regions from which the skull samples were taken.

Magnetic measurements

The magnetic mass susceptibility, χ, of the samples was measured with a Faraday balance, which has a resolution of 10^{-10} emu g^{-1}. The susceptibility was measured at room temperature and in magnetic fields, H, ranging from 0.9 to 8.5 kOe. The samples were mounted in a little bucket made of an

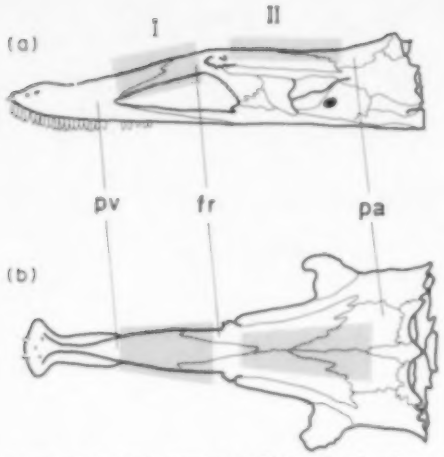

Fig. 1. Skull of *Anguilla japonica*. (a) Shows the view from the side, (b) from above. pv, premaxilloethmovomerine block; fr, frontale; pa, parietale (redrawn form Matsui and Takai, 1959). Our samples from eel skulls were taken either from region I or II.

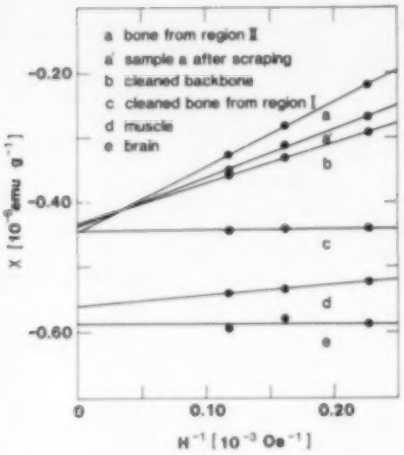

Fig. 2. The mass susceptibility of six samples of silver eels (*Augilla anguilla* L.) vs inverse magnetic field. The straight lines are the regression lines. For the brain sample an additional point obtained in a lower field was included in the adjustment because of the higher relative inaccuracy in these measurements. The mass of the brain was 13 mg, of the muscle (taken from above the skull roof) 30 mg and of the other samples about 20 mg.

aluminium alloy and all measurements were corrected for the contribution of the sample holder. The force on a sample is proportional to its mass and with a constant uncertainty in a force measurement this yields a larger relative inaccuracy the smaller the sample and the lower the magnetic field. For a sample of 30 mg the uncertainty in the calculated high field susceptibility is estimated to be less than $\pm 1\%$ whereas for a 10 mg sample it may amount to a few per cent. Further details of the apparatus and calibration may be found in Dellby and Ekström (1971) and Hanson (1978).

RESULTS

We have investigated about 20 different samples from seven eels. All the samples are diamagnetic and some of them exhibit a small field dependence in their magnetic susceptibility. This is exemplified in Fig. 2, where the susceptibility of six samples, representing different tissues, is plotted vs the inverse field. Usually the field dependence is small and the susceptibility is linear in H^{-1} for high fields. Thus it may be accounted for by the presence of minute amounts of ferro- or ferrimagnetic precipitates, whose magnetization becomes saturated in high fields. The slope in the linear region is a measure of the amount of magnetic material in the sample and its concentration, c_m, may be estimated by adjustment of the relation

$$\chi = \chi_0 + c_m \sigma_s / H \qquad (1)$$

to the experimental points. σ_s is the saturation magnetization of the precipitates. The intercept with the axis, χ_0, yields the diamagnetic susceptibility of the matrix. The results obtained from this adjustment are given in Table 1.

Table 1. Compilation of magnetic data for samples from silver eels (*Anguilla anguilla* L.)

Sample	Localization	Individual No.	Number of samples	χ_0 10^{-6} emu g^{-1}	$c_m\sigma_s$ 10^{-3} emu g^{-1}
Brain		1 2	2	−0.59	0.0
				−0.59	0.0
Tissue	Above skull roof	2	1	−0.56	0.2
Tissue	Scraped from ventral side of skull roof	5	1	−0.58	0.6
Bones	From parietale, frontale and back of premaxilloethmovomerine block, region II in Fig. 1	1 2	5	−0.47 ± 0.04	1.0 ± 0.2
Bones	From premaxilloethmovomerine block, region I in Fig. 1	7	2	−0.45	0.6
				−0.46	0.8
Bones	From pectoral girdle	3	1	−0.42	0.6
Bones	From vertebral column at height of pectoral fin	4	1	−0.48	0.7
Bones boiled and scraped	From premaxilloethmovomerine block, region I in Fig. 1	5 6	6	−0.46 ± 0.03	0.01 ± 0.05
Bones boiled and scraped	From vertebral column immediately posterior to the skull	5	2	−0.43	0.6
				−0.41	1.0

The diamagnetic background, χ_0, and the saturation magnetization, $c_m\sigma_s$, were obtained by linear regression of χ vs H^{-1}. The adjustment was made using the points for three values of the field, 4.4, 6.2 and 8.5 kOe. The mean value and standard deviation are given when the data represent more than two samples.

All bone samples, as dissected, contain magnetic material. The periosteum is included in the samples and in those taken from the skull the meninx as well. Some of the samples were further investigated after treatment in an attempt to separate the bones from the attached membranes. This was done by scraping, in some cases after boiling in distilled water. As the pieces were cleaned the amount of magnetic material was decreased. This is exemplified in Fig. 2 with a sample of bone from region II in the skull, that was measured before and after scraping. The diamagnetic background was not significantly changed by the cleaning procedure. The only samples of bone where the magnetic precipitates could be completely removed were taken from region I in the skull. We could not get any samples of the vertebrae or from region II in the skull that were free of magnetic material. This may possibly be explained by the difficulty to scrape these bones which are thin with many joints and uneven surfaces. A sample of the tissue that was scraped off from the ventral side of the skull was also tested and found to have a magnetic content.

All bone samples yield the same adjusted χ_0 which also coincides with the diamagnetic susceptibility of the field independent samples. Thus we conclude that the eel bones have a diamagnetic susceptibility given by the mean value and the standard deviation

$$\chi_{bone} = -(0.45 \pm 0.02) \cdot 10^{-6} \text{emu g}^{-1}.$$

This value was obtained from 22 independent measurements on the samples in Table 1 including results after scraping.

The samples of soft tissues are all more strongly diamagnetic than the bones. The brain is completely free of magnetic precipitates.

DISCUSSION

When studying magnetic material in such dilutions as found in the biological tissues, one has to be extremely careful not to contaminate the samples.

We feel confident that there is magnetic material in connection to the bones in eel for the following reasons

- our sample preparation yielded reproducible results for different individual eels;
- we found magnetic material in all dissected bone samples;
- all samples which mainly contain bone yield the same diamagnetic background.

We do not know what kind of substance this magnetic material is. Atoms or ions carrying magnetic moments can be looked for among the 3d-metals or rare earths. These metals are found as trace elements in biological material with iron being by far the most abundant. Mammal bones, for instance, have been reported to contain 200 ppm Fe, < 3.5 ppm Ni, < 0.85 ppm Cr and 0.19 ppm Mn of the dry wt and still much smaller amounts of any other potentially magnetic element (Bowen, 1966). High iron contents can be found in bone marrow, red blood cells, liver and spleen. Essential functions of the iron compounds are as oxygen carriers (hemoglobin, myoglobin), enzymes and iron stores (ferritin). In these cases one or several Fe-ions are bound to large or small organic molecules where the iron invariably yields a paramagnetic contribution to the magnetic susceptibility. Although we find it most reasonable to believe that the magnetic content in our samples stems from iron it cannot be in the usual form as in the metalloproteins or iron complexes. Our susceptibility data give evidence of magnetic precipitates or particles. A magnetic substance known to be biochemically precipitated is magnetite Fe_3O_4. If we assume that our samples contain magnetitie, with a saturation magnetization $\sigma_s = 92$ emu g^{-1} (Smit and Wijn, 1959) the susceptibility data would yield magnetite concentrations of the order of 10 ppm of dry wt or less. This does not give an excess of iron content as compared with the chemically analysed concentration of iron in other fish. Plaice. (*Pleuronectes platessa* L.) for instance contains 22 ppm iron of the wet wt in bone and 6 ppm of the wet wt in muscle (Pentreath, 1973).

No direct comparison can be made between these data and other reports on magnetic material, deduced from magnetic remanence, in vertebrates. From our saturation magnetization values we get the total amount of those precipitates that are large enough to be saturated in a field of 4.4 kOe at room temperature. This includes 'magnetically stable' particles which may exhibit remanence as well as smaller, superparamagnetic particles. A measurement of the remanence, natural or induced, in our samples would yield a smaller estimate of the amount of magnetic material. We can however make a comparison concerning the order of magnitude of for instance the remanence in pigeons (Walcott *et al.*, 1979) and the magnetization in one of our samples. With a value of $c_m\sigma_s = 1.0 \cdot 10^{-3}$ emu g^{-1} (cf. Table 1) a sample of 20 mg has a saturation magnetization of $2 \cdot 10^{-5}$ emu. This is the same as the induced remanence found in the pigeons. The location in the pigeon was "either in a small (1 by 2 mm) piece of tissue between the dura and the skull or was too closely associated with the skull to be separated from it conveniently". Similar amounts of induced remanence ($10^{-6} - 1.5 \cdot 10^{-5}$ emu) were also found in pigeons (*Columba livia*) and white-crowned sparrows (*Zontrichia leucophrys*) by Presti and Pettigrew (1980). The remanence in their pigeons was found in the head where it "was diffuse, being spread rather uniformly throughout the entire skull" and in the neck musculature. Natural remanence was found in the head of the common Pacific dolphin (*Delphinus delphis*) by Zoeger *et al.* (1981). All sections of the head were detectably magnetized, but with one section more magnetic than the others. One of the most magnetic samples, a few mm^3 of tissue taken from the dura mater, contained $2 \cdot 10^{-5}$ emu. In humans, induced magnetic remanence was found in bones from the region of the sphenoid/ethmoid sinus complex (Baker *et al.*, 1983).

The eel with a single meninx is anatomically different both from the pigeon with two and mammals with three meninges. Still, we think that the locations of the magnetic material in the head of eels and other species are equivalent, with respect to the bone. Also the amounts reported are comparable with what we find in the eels. The remanence results showing high concentrations of magnetic material in relatively small volumes do not necessarily contradict measurements implying a more diffuse localization. Only magnetic precipitates large enough to have an anisotropy energy comparable to the thermal energy contribute to the

remanence. Furthermore, unless a sample has been magnetized to saturation, the induced remanence depends on the strength of the field to which the sample was exposed. Thus a variation in the magnetic remanence over the tissue volume may equally well be explained by fluctuations in the particle sizes as by a variation in the concentration of the magnetic material.

However, in the eels we also find magnetic material in connection to bones other than in the skull. It is not clear to what extent this may be the case for the other species as well, since here the investigations were concentrating upon the heads. In this connection we consider it important to stress that no conclusions as to the uniqueness of the findings of magnetic material in the skull should be drawn until other parts of the animal's body have been examined as well. Up to date there is a single report where the entire body of a vertebrate species has been examined. Quinn *et al.* (1981) measured the magnetic remanence, natural and induced, in sockeye salmon fry. They found no detectable ($< 2 \cdot 10^{-7}$ emu) magnetic material in 26 out of 30 fry and the magnetic material in the other four was attributed to environmental contamination. As was argued above this does not exclude the presence of smaller, superparamagnetic particles. Thus we cannot, at this moment, decide whether eel and salmon are different with respect to magnetic material content.

Our main results may be summarized as follows. The European eel (*Anguilla anguilla* L.) has magnetic material in connection to the bones. In samples taken from the premaxilloethmovomerine block the magnetic material is located in the periosteum and/or the meninx (or possibly in the outermost layer of the bone) and the bone yields a purely diamagnetic background. We have not shown that the magnetic material is strictly localized to the periosteum or the meninx for the other bones, since these could not be properly separated from the attached membranes.

The localization and the amount of the magnetic material in the head of the eels is comparable with findings in other vetebrates.

We do not yet know what kind of substance this magnetic material is nor its function. We have begun further investigations of the skull tissue starting by microscopy studies, that will hopefully shed some light upon this question.

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INTRASOMATICALLY RECORDED ACTION POTENTIALS IN SNAIL NEURONS (*HELIX POMATIA*): DIFFERENT SHAPES WITH DIFFERENT SITES OF ORIGIN IN THE NEURONAL ARBORIZATION. A COMBINED MORPHOLOGICAL AND ELECTROPHYSIOLOGICAL STUDY

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Abstract—1. Fibres of the identified neurons B1 to B3 in the buccal ganglia of *Helix pomatia* can be divided into three types according to their diameters.
2. Electrical stimulation of nerves containing the different fibres induces typical fast depolarizations in the somata of neurons B1 to B3. The appearance of these depolarizations is strictly correlated to the fibre types.
3. The depolarizations are interpreted as axonal action potentials.

INTRODUCTION

With a microelectrode in the soma of a neuron and electrical stimulation of peripheral fibre bundles responses have often been recorded which contained fast depolarizations. These depolarizations have been reported to be all-or-nothing events, to be of small amplitudes (*ca* 1 mV) and to show short on-set latencies (Spencer and Kandel, 1961; Blankenship and Kuno, 1968; Llinas and Nicholson, 1971; Llinas *et al.*, 1971, 1974; Czeh, 1972; Mellon, 1972; Korn *et al.*, 1973; Schwartzkroin, 1977; Sonnhof *et al.*, 1977; Gutnik and Prince, 1981). They have been called "fast prepotentials" (Spencer and Kandel, 1961), "short latency depolarizations" (Baker and Llinas, 1971), "graded antidromic depolarizations" (cf. Korn *et al.*, 1973), "ventral root EPSP" (cf. Sonnhof *et al.*, 1977) and "early depolarizing potentials" (cf. Precht, 1975). However, the elementary mechanisms underlying these depolarizations are obscure in many cases. In accordance to literature they might represent monosynaptically induced electrotonical excitatory postsynaptic potentials (EPSP) or antidromic action potentials (AP) the conduction of which being blocked in the neuronal arborization. The distinction between both alternatives proved to be difficult applying electrophysiological techniques only (Bennett, 1966; Mellon, 1972; Altrup *et al.*, 1979). Therefore, morphological and electrophysiological techniques have been combined to contribute to a principle interpretation of such fast depolarizations. In these investigations evidence could be accumulated that in the investigated nervous system used as a model many fast depolarizations represent blocked antidromic AP of thin fibres of the impaled neuron.

MATERIALS AND METHODS

The experiments were carried out on the identified neurons B1, B2 and B3 in the buccal ganglia of *Helix pomatia* (Cottrell and Macon, 1974; Schulze *et al.*, 1975). The ganglia were isolated from the animal and fixed in a chamber continuously perfused with a solution containing NaCl: 130; KCl: 4.5; $CaCl_2$: 9; and Tris–Cl: 5 mmol/l.

Intracellular stainings were performed according to the method described by Altrup and Peters (1982). In principal, it comprised pressure injection of a cobalt–lysine dye, precipitation of cobalt–sulphide by ammonium sulphide, silver intensification and whole mount embedding. The staining technique enables even very small fibres (diameters below 1 μm) to be identified in whole mount preparations.

The membrane potential was recorded by microelectrodes filled with a 3 mol/l KCl or a 3 mol/l Na–acetate solution. The recordings were taken from the somata of the neurons. Shifts of the membrane potential were induced by current injection via a second intracellular microelectrode.

For stimulation, the ipsi- and contralateral nerves were routinely sucked into glass pipettes connected to the stimulator by Ag/AgCl wires. In most cases, the nerves were stimulated against a circular Ag/AgCl bath electrode which was fixed to the wall of the experimental chamber. In control experiments Ag/AgCl ball electrodes served as a reference. They were placed on the nerves immediately outside the suction electrodes. The current pulses were negative/positive and had a duration of 3 msec.

For evaluation, the neuronal responses of nerves to stimulation were photographed from an oscilloscope or, in most cases, digitally interstored and then written out slowly on a pen recorder.

RESULTS

Principles and types of neuronal arborizations are presented at first. In subsequent chapters, responses are described which were elicited in neurons B1 to B3 by electrical stimulation of nerves with and without fibres of the recorded neurons.

Morphology and topography of neuronal arborizations

The neuronal arborizations were visualized by intracellular staining. An example of a stained neuron is given in Fig. 1(A). Cobalt–lysine was injected into the soma. After *ca* 1 hr the dye was transported into the neuronal arborization located in the neuropil and in the nerves.

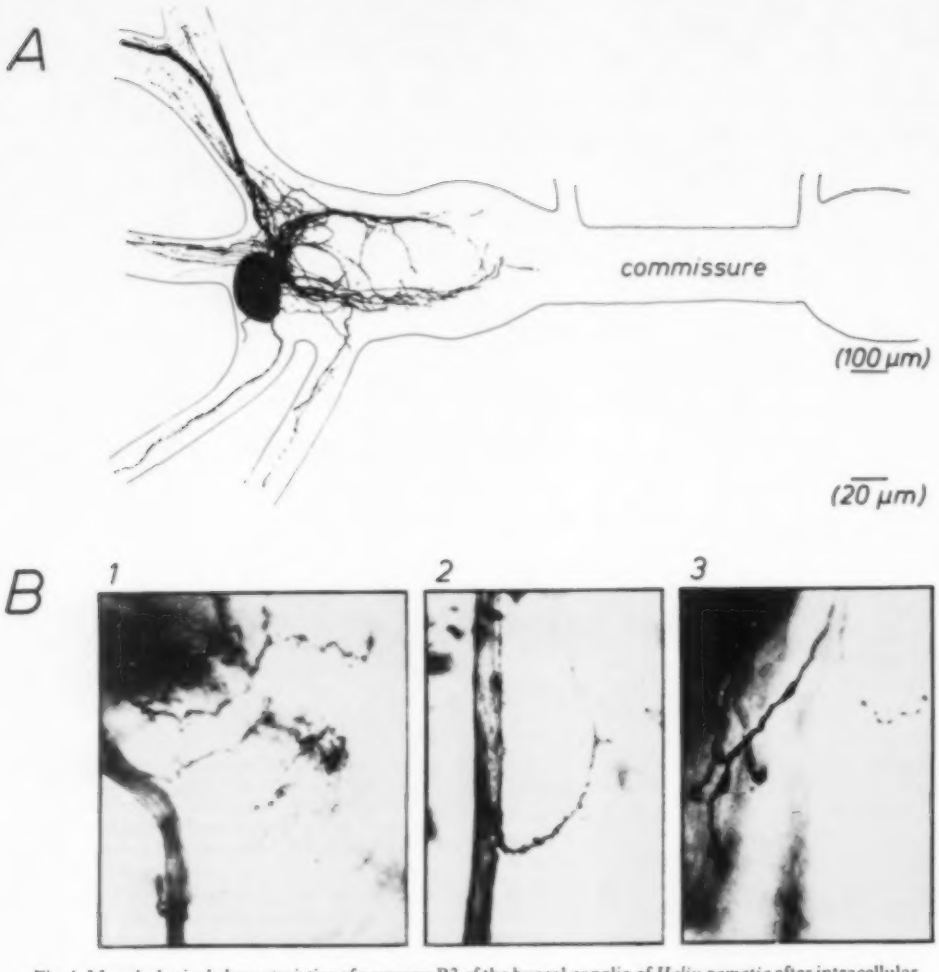

Fig. 1. Morphological characteristics of a neuron B3 of the buccal ganglia of *Helix pomatia* after intracellular staining with cobalt–lysine. (A) Drawing of the neuron after whole mount embedding. (B) Photographs of details of intracellularly stained neurons B3 showing the main axon with axon collaterals (1, 2) and fibre terminations in the neuropil (3). Calibrations not corrected for shrinkage.

From stainings as presented in Fig. 1 it can be derived: (i) The dye was not transported through known electrical contacts. Thus, the ipsi- and contralateral neurons B2 were found to be coupled electrically with each other with a coupling coefficient of *ca* 1:10 (Altrup and Speckmann, 1981) and the staining of one neuron B2 did not co-stain the contralateral cell of this type. (ii) The fibres could be followed microscopically from their origin to their termination. They started always from the so-called main axon (Fig. 1, B1 and 2) and ended in short swellings (Fig. 1, B3). (iii) The branchings were found to be stained without abrupt change in dye distribution. As a whole, from these findings the co-staining of fibres of other neurons is unlikely.

In the neurons B1, B2 and B3, three types of fibres can be distinguished according to their diameters. That is demonstrated at hand of neuron B3, a staining of which is presented in Fig. 2(A): (1) One or more main axons (diameters 10–20 µm) are located in the ipsilateral cerebrobuccal connective. (2) Middle-sized fibres (diameters 2–5 µm) enter one of the ipsilateral salivary gland nerves. (3) Thin fibres (diameters *ca* 1 µm or less) are regularly found in the neuropil and in the ipsilateral 1 and 2 pharyngeal nerves. Concerning the statement of fibre diameters, the shrinkage of the alcohol-fixed preparations has not been considered.

Stimulation of nerves containing fibres of the recorded neuron

In further experiments it was tested which of the morphologically differentiated types of fibres are able to generate AP. This could be done by electrical stimulation since the fibres of different calibres were found to run in distinct nerves. A typical experiment is shown in Fig. 2. Prior to the intracellular staining of the neuron, the nerves of the buccal ganglia were stimulated subsequently and the neuronal responses were recorded with an intrasomatic microelectrode. Considering the first parts of the neuronal responses, three types of fast depolarizations can be distinguished. They are labelled a, b and c in Fig. 2(B). The

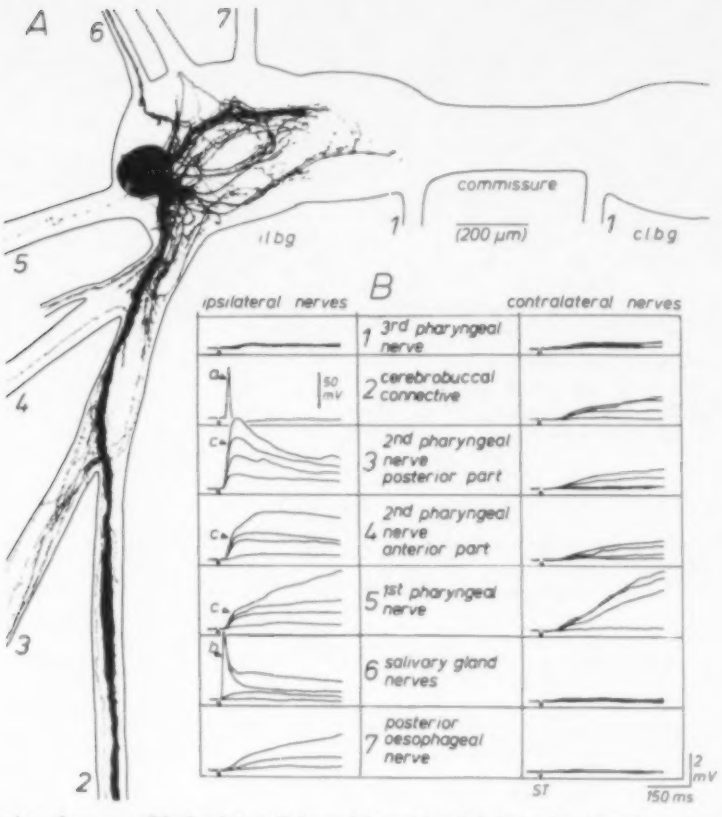

Fig. 2. Drawing of a neuron B3 after intracellular staining with cobalt-lysine in a buccal ganglion of *Helix pomatia* (A) and responses of the membrane potential of the neuron in A to electrical stimulation of nerves of the buccal ganglia (B). (A) The neuron is drawn after whole mount embedding in the contour of the ganglia and nerves. i.l.b.g. and c.l.b.g.: Ipsilateral and contralateral buccal ganglion. Calibration is not corrected for shrinkage. The nerves are labelled 1 to 7 corresponding to the numbers in B. (B) The responses of the membrane potential to stimulation of ipsilateral and contralateral nerves are arranged in a left and a right column. Upward deflection: Depolarization. Responses evoked with increasing stimulus intensities are superimposed. The stimuli (ST) are marked by dots. The stimulated nerves are indicated in the middle column. The calibrations in line 7 refer to all recordings except those in line 2, left column. Types of fast depolarizations are labelled a, b and c.

stimulation of a main axon (Fig. 2, A2) evoked full somatic AP named "a" in Fig. 2 (B2). With decreased excitability of the soma, e.g. during repetitive stimulation or hyperpolarization, the full AP were replaced by depolarizations which showed amplitudes of 10–50 mV and represented obviously the known axonal AP (Tauc and Hughes, 1963). The stimulation of a nerve containing a middle-sized fibre of the recorded neuron (Fig. 2, A6) induced fast depolarizations of amplitudes of 2–5 mV ("b" in Fig. 2, B6). They resembled small axonal AP (Westerfield *et al.*, 1978). Stimuli applied to nerves which were entered by thin fibres (Fig. 2, A3, 4 and 5) evoked also fast depolarizations which are assigned "c" in Fig. 2 (B3, 4 and 5). Their on-set latencies were similar to those of response types a and b and their time-to-peak only slightly exceeded the duration of a full somatic AP. Their amplitudes depended upon the number of thin fibres in the stimulated nerve. When there was only one thin fibre, the depolarization had a constant amplitude of *ca* 1 mV. In this case the fast depolarization showed an all-or-none characteristic with increasing stimulus intensity. The final amplitude of the depolarization was higher the more fibres entered the stimulated nerve. In all experiments, the depolarizations of types a, b and c were strictly correlated to the fibre types 1, 2 and 3, respectively.

The described fast depolarizations showed the following further properties: (1) In a given preparation, the on-set latency was constant with single identical stimuli, with repetitive stimulation, and with increasing stimulus intensity. (2) The fast depolarizations showed a threshold characteristic. In some cases they increased by steps with increasing stimulus intensity. (3) Their amplitudes were not markedly affected when the resting membrane potential (RMP) of the investigated neuron was increased beyond −50 mV. However, at higher levels of RMP the depolarizations were blocked (see also: Altrup and Speckmann, 1982). (4) An increase in the extracellular Ca-concentration (40 mmol/l) which has been found to depress interneuronal activities (Cottrell and Macon, 1974) did not block these depolarizations. (5) Extracellularly applied proteolytic enzymes (Pronase, 1% for 5 min) which

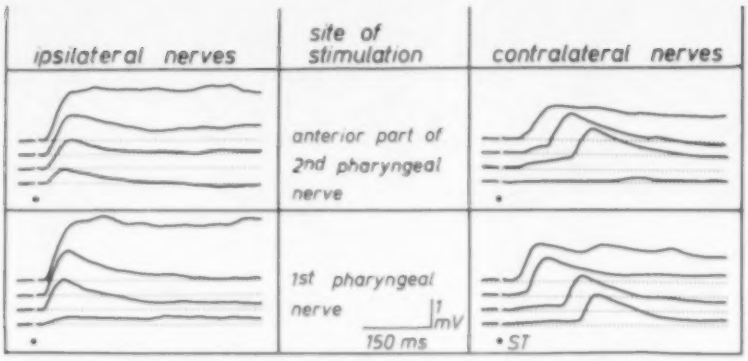

Fig. 3. Responses of the membrane potential of a neuron B3 in the buccal ganglia of *Helix pomatia* to the stimulation of ipsilateral (left part) and contralateral nerves (right part). Upward deflection: Depolarization. Responses evoked with increasing intensities of stimulation were superimposed. The stimuli (ST) are marked by dots. The stimulated nerves are indicated in the middle.

have been described to decrease most chemically mediated excitatory post-synaptic potentials irreversibly to zero levels (Altrup *et al.*, 1980) did not affect the fast depolarizations.

In summary, the findings suggest that the fast depolarizations represent axonal AP of the impaled neuron.

Stimulation of nerves containing no fibres of a recorded neuron

The stimulation of nerves which contained no fibres of the simultaneously recorded neuron did not evoke fast depolarizations of short on-set latencies. However, fast depolarizations of long and variable on-set latencies appeared in some preparations (type d depolarizations). Typical examples are displayed in the right part of Fig. 3. For sake of comparison, fast depolarizations of short latencies (type c depolarizations) are displayed in the left part of the figure. Comparing both types of depolarizations, most of their properties are almost identical. However, whereas the type c depolarizations were the first neuronal responses to the stimulation of a nerve, the type d depolarizations occurred superimposed on EPSP. Furthermore, whereas the on-set latencies of type c depolarizations proved to be constant, the latencies of type d depolarizations decreased with increasing intensity of stimulation (cf Fig. 3) and increased with increasing RMP.

The observations suggest that the depolarizations of type d represent axonal AP of the impaled neuron which are induced by synaptic activation.

DISCUSSION

The results are summarized and interpreted in Fig. 4. In the upper part of the figure typical examples of fast depolarizations are displayed. The depolarizations of types b and c can be assumed to represent axonal AP considering their electrophysiological properties as well as their strict relation to the stimulation of fibres of types 2 and 3. The fast depolarizations of type d may also be axonal AP. They might be evoked via synaptic transmission, since they were elicited by stimulation of nerves without a fibre of the impaled neuron.

Interpreting the fast depolarizations as axonal AP it must be assumed that the propagation of those of types b and c is blocked in the neuronal arborization (cf. also: Spencer and Kandel, 1961; Llinas and Nicholson, 1971; Mellon, 1972; Traub and Llinas, 1979). The elementary mechanism of this block will be discussed at hand of the schematic drawing in Fig. 4. The stimulation of the middle-sized fibre (ST b) and of the thin fibres (ST c) evoke AP which run to the main axon. There an abrupt decrease in current density occurs due to the abrupt increase in fibre diameter. Consequently, it is possible that axonal AP fail to depolarize the membrane of the main axon to a critical level (cf. Mellon, 1972). Recording the membrane potential from within the soma the presumed AP were picked up from the branching point. The amplitudes of the potentials recorded in the soma are in agreement with the above described assumptions since they correspond to the ratios of diameters of the stimulated fibres to the main axon (cf. Harley, 1978).

In general, the presumed fibre-AP recorded in the neuronal soma are hardly differentiated from electrotonical EPSP since both depolarizations show the same changes with experimental parameters (Altrup *et al.*, 1979). Thus, directly evoked fibre-AP correspond to monosynaptically mediated electrotonical EPSP, and synaptically evoked fibre-AP share their properties with oligo/polysynaptical EPSP. Both depolarizations can increase in amplitude with increasing intensity of stimulation provided the stimulated nerve contains several fibres of the impaled neuron or several electrically coupled axons of other neurons. Thus, the differentiation of fibre-AP and electrotonical EPSP is difficult in many cases (Bennett, 1966; Altrup and Speckmann, 1982). The present paper shows that the probability of identification of fibre-AP increases when the investigated neuron is stained intracellularly in order to show whether or not there are fibres of the neuron within the range of the stimulating electrode. In this context observations should be mentioned which may facilitate the identification of electrotonical EPSP in some cases. Thus, electrically mediated responses, identified by simultaneous recordings from the coupled neurons, often consisted of a biphasic positive/negative fluctuation representing the depolar-

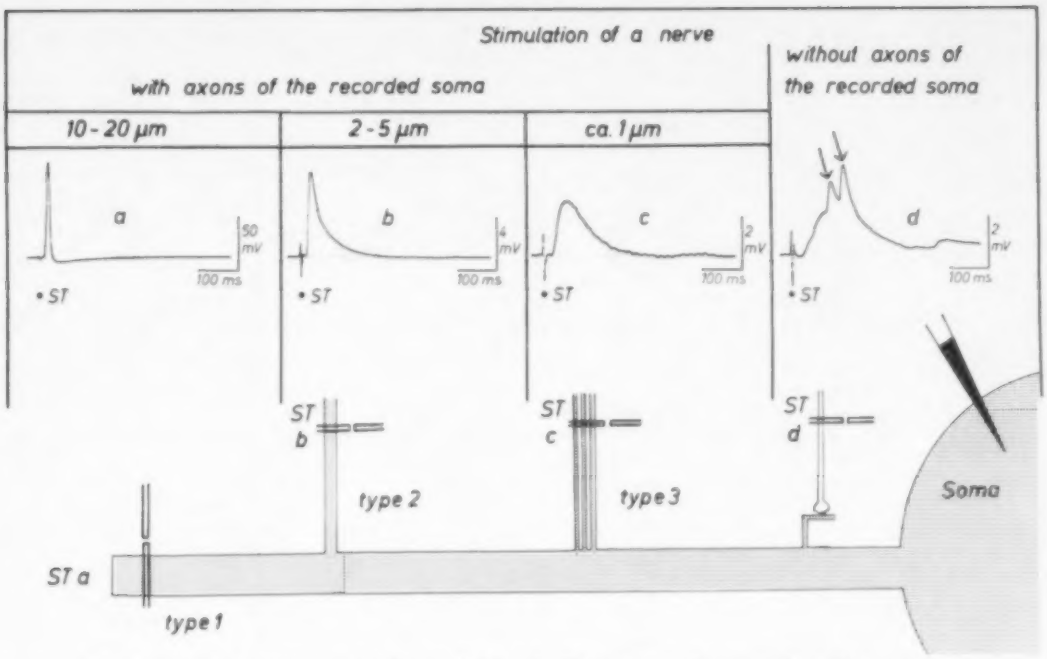

Fig. 4. Suggested elementary mechanisms underlying the different types of responses (a to d) in neurons B1, B2 and B3 of the buccal ganglia of Helix pomatia. The responses a to d are assumed to be evoked by the stimulation of nerves containing a main axon (St a), a middle-sized axon (St b), thin fibres (St c) and of nerves without fibres of the impaled neuron (St d). Type 1 to 3 refer to fibres of different diameters. The stimuli (ST) are marked by dots.

ization of the AP and the after hyperpolarization of the discharging neuron. Such biphasic fluctuations were never observed in cases in which fibres of the investigated neuron were found in the stimulated nerve. Consequently, the occurrence of biphasic electrotonical fluctuations leads one to suppose that they represent EPSP whereas monophasic electrotonical depolarizations are furthermore either fibre-AP or EPSP (Altrup *et al.*, 1979; Altrup and Speckmann, 1982).

The functional significance of the assumed fibre-AP described in the present paper is still obscure. They might represent a second stage of input integration between the post synaptic potentials and full somatic AP. Furthermore, it should be taken into account that their function consists in the reset of ongoing EPSP in a single fibre, or in the accentuation of EPSP generated far from the AP initiating zone (Eccles *et al.*, 1958; Maekawa and Purpura, 1967) or in the affection of the metabolism of selected fibres (Schmitt, 1979).

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UPTAKE AND DISTRIBUTION OF THE AMINO ACID LEUCINE BY FRESHWATER TRICLADS IN DIFFERENT REPRODUCTIVE STATES

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Abstract—1. The distribution of labelled leucine to the tissues of mature, sexual triclads was non-random, the greatest incorporation being by the reproductive system and particularly the vitelline tissues.

2. Incorporation into the reproductive system was more marked in semelparous (breed once and die) than in iteroparous (breed repeatedly over several seasons) triclads.

3. No antero-posterior gradients of uptake and incorporation were discovered in either mature sexual or immature sexual or asexual triclads.

INTRODUCTION

In a previous paper (Calow and Read, 1981) we reported the occurrence of transepidermal uptake of isotopically labelled amino acids by adult, sexual, freshwater triclads (Platyhelminthes). The subsequent distribution of these to the tissues was extensive and non-random but did not conform to the antero-posterior gradient of distribution reported for some asexual triclads (Coward and Flickinger, 1965). In the sexual triclads, labelled amino acids became aggregated in the gonads and ovo-vitelline tissues and we suggested that this might have obscured any underlying gradients. Here, using autoradiographic techniques, we compare the uptake and distribution of [^{14}C]leucine by immature and mature sexual triclads and by asexual *Polycelis felina* (Calow *et al.*, 1979). Two sexual species were considered, *Dendrocoelum lacteum* and *Polycelis tenuis*. The former is semelparous (breeds once and dies) and annual and the latter iteroparous (breeds repeatedly over several seasons) and perennial (Reynoldson, 1978). Previous work on the metabolism of these two species (Calow and Woollhead, 1977; Woollhead and Calow, 1979) has suggested that the semelparous triclad invests more resources in reproduction than the iteroparous one and so we also investigated the effect of this on the differential uptake of labelled amino acids into the somatic and reproductive systems of both species.

MATERIALS AND METHODS

Triclads were collected from the field, acclimatized (10°C, no food, continuous darkness) for 5 days and then exposed to [^{14}C]leucine for 24 h. Administration of the isotope and dose levels were as described in Calow and Read (1981). Juvenile *Dendrocoelum lacteum* and *Polycelis tenuis* were collected between August and September when their reproductive systems (testes, ovaries and vitelline glands) were undifferentiated. Mature specimens, with differentiated reproductive organs, were collected between December and April. Specimens of *Polycelis felina* were collected from a population known to be exclusively asexual (Calow *et al.*, 1979) and, upon histological examination, showed no traces of gonads or ovo-vitelline tissues.

Autoradiographic techniques were as described in Calow and Read (1981). The antero-posterior distribution of label was investigated by making longitudinal transects, with the number of grains per 900 μm^2 being counted at 150 μm intervals. For mature *P. tenuis* several transects were taken at 200–300 μm intervals from the edge to the middle of the worm. In *D. lacteum* a single transect was taken approximately halfway between the middle and the edge of the triclad, since previous results suggested that activity peaked in this region (Calow and Read, 1981). The same was assumed to be true of *P. felina* and, here, only a single transect, halfway between the edge and middle of the triclad, was considered.

To investigate the relative distribution of label between somatic and reproductive tissues, 30 random samples (900 μm^2) were counted in each tissue from > 10 randomly chosen sections.

Two to five triclads were used to investigate the antero-posterior distribution of grains in *D. lacteum* and *P. tenuis*, but individual triclads were used in all other determinations.

RESULTS

Lateral and longitudinal distribution in P. tenuis

Figure 1 shows the distribution of grains along antero-posterior transects of a mature *P. tenuis* taken at different distances from the lateral edge of the triclad. Visual inspection suggests no obvious antero-posterior gradients in any of the transects. The means and confidence limits ($\pm$2SE) for each transect are presented at the end of each graph, and analysis of variance (completely randomized design) indicated that significant differences occurred in these data ($F = 17.7$ for 5/171 *df*, $P < 0.05$). Using the criterion that the differences between individual means must be greater than the sum of their confidence limits to be significant ($P < 0.05$) it can be seen that the lateral margin had significantly lower counts and the central transect (i.e. halfway between the edge and the middle of the triclad) had significantly higher counts than the other transects. These results are similar to findings from *D. lacteum* (Calow and Read, 1981), and all

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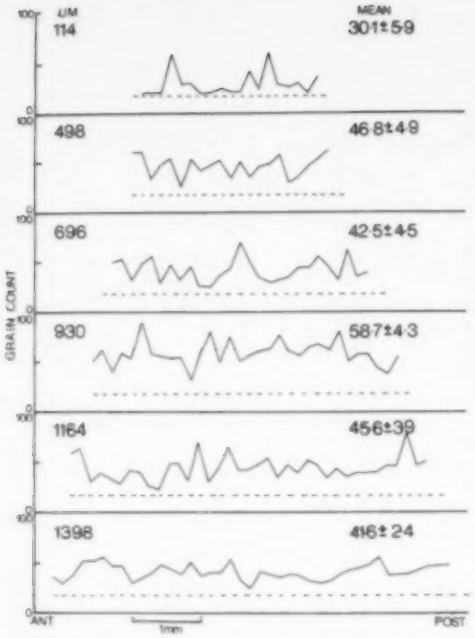

Fig. 1. Density of grains in serial autoradiographs of an adult *P. tenuis*. Broken lines denote background. Numbers to the left are distances of transects from the lateral margin of the triclad and numbers to the right are mean (±2SE) grain counts per 900 μm^2.

subsequent analyses of the longitudinal distribution of counts will be restricted to transects taken halfway between the edge and the middle of the triclads.

Longitudinal distribution in mature, immature and asexual triclads

Figure 2 shows autoradiographs of whole, immature (A) and mature (B) *P. tenuis*. There was no obvious antero-posterior gradient of grains in either specimen, though the grain density in the posterior tip of the immature specimen appeared to be higher than the other tissue. The distribution of grains was more patchy in the mature than the immature triclad, and patches of high activity in the former corresponded to ovo-vitelline tissues and testes. Three to four points of high density of grain on the dorsal, anterior tip of this specimen are ectosymbiotic protozoans.

Samples from the transects were divided into three equal groups, representing anterior, middle and posterior portions of the triclads. These data are presented in Table 1 for individual triclads. Analysis of variance indicated that, apart from in mature *D. lacteum*, there were no significant differences in the density of grains in the different sections of any triclads. In the mature *D. lacteum*, three out of four triclads showed significant differences, two with the posterior portions showing higher counts than the rest and one with the anterior portion having the highest count. Immature *P. tenuis* had higher counts than mature specimens, but there was no obvious difference between the age-classes of *D. lacteum*.

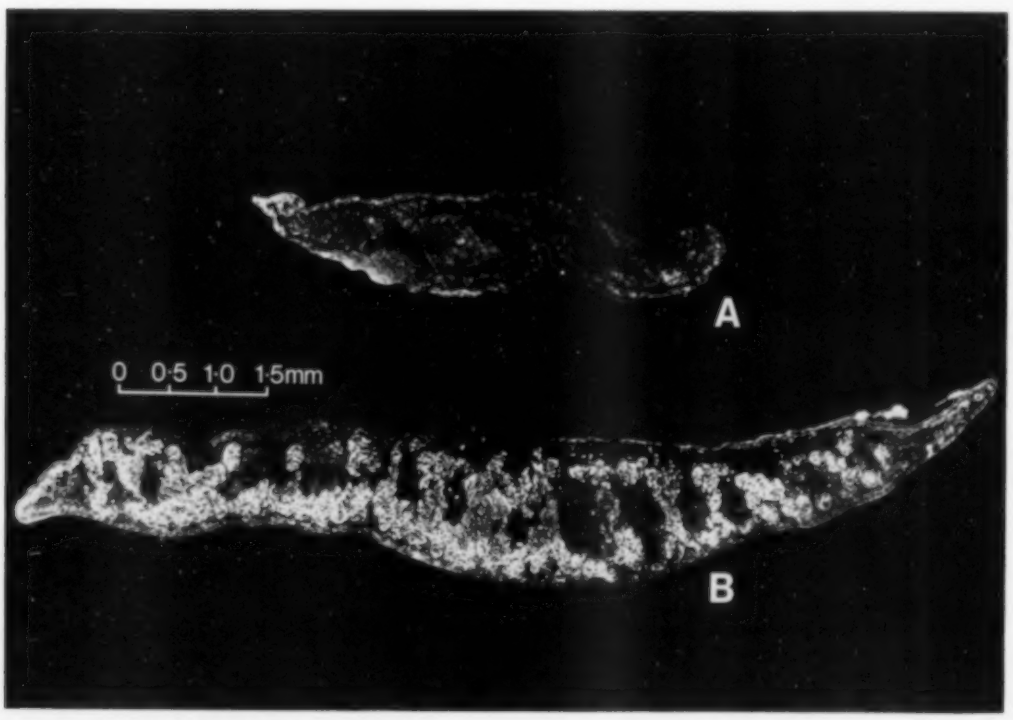

Fig. 2. Autoradiographs of (A) juvenile (without reproductive system) and (B) mature (with reproductive system) *P. tenuis*. Anterior is to the right and posterior to the left.

Table 1. Density of grains (per 900 μm^2) in different portions of triclads

Species	Mean counts ± 2SE Anterior	Middle	Posterior	F*	df*	P*
Mature *P. tenuis*	72.3 ± 8.7	60.0 ± 16.3	66.5 ± 14.9	0.80	2/31	> 0.05
	30.0 ± 14.0	33.9 ± 7.0	34.7 ± 9.5	0.23	2/31	> 0.05
	27.0 ± 10.9	24.9 ± 14.0	26.8 ± 9.3	0.04	2/36	> 0.05
	38.9 ± 5.8	32.2 ± 10.6	41.3 ± 10.9	0.99	2/36	> 0.05
Immature *P. tenuis*	100.0 ± 12.6	118.5 ± 25.8	104.8 ± 13.3	1.11	2/25	> 0.05
	138.9 ± 39.1	98.6 ± 15.8	142.3 ± 25.3	2.93	2/20	> 0.05
	78.3 ± 29.4	87.4 ± 19.1	113.5 ± 55.7	0.80	2/20	> 0.05
	69.9 ± 46.0	76.8 ± 19.2	127.9 ± 35.4	2.89	2/20	> 0.05
	76.8 ± 23.0	57.8 ± 12.2	72.5 ± 31.9	0.70	2/21	> 0.05
Mature *D. lacteum*	59.1 ± 12.0	41.6 ± 11.4	72.1 ± 13.5	6.06	2/52	< 0.05
	24.5 ± 5.5	21.7 ± 4.8	35.4 ± 8.8	4.77	2/81	< 0.05
	21.3 ± 8.2	14.7 ± 2.9	26.9 ± 9.0	2.73	2/90	> 0.05
	36.3 ± 8.6	17.8 ± 4.4	21.9 ± 6.6	8.09	2/88	< 0.05
Immature *D. lacteum*	50.9 ± 14.9	59.0 ± 11.4	55.8 ± 21.4	0.23	2/20	> 0.05
	32.2 ± 10.0	24.0 ± 5.2	30.4 ± 12.0	0.82	2/62	> 0.05
P. felina	22.3 ± 8.4	22.2 ± 7.8	21.0 ± 10.2	0.03	2/18	> 0.05

*F = variance ratios; df = degrees of freedom; P = probability level.

There was no significant difference between counts in the different portions of the single *P. felina* that was examined. Other specimens were observed without making any quantitiative estimations and confirmed this interpretation.

Distribution between somatic and reproductive tissues

The distribution of counts between somatic and reproductive tissues in specimens of *D. lacteum* and *P. tenuis* is summarized in Table 2. The testes of *D. lacteum* regress before breeding starts whereas all the reproductive organs of *P. tenuis* persist throughout the breeding season (D. Read, in preparation). Hence two counts are given for *D. lacteum*; one for a specimen collected early in the year and with testes (December), the other for a specimen in which testes had disappeared (April). Also given in Table 2 are ratios of counts in the reproductive tissues to those in the somatic tissues.

Analyses of variance indicated significant differences in counts between tissues in all specimens. In *Dendrocoelum lacteum*, reproductive tissues had higher counts than somatic and highest counts were found in the ovo-vitelline tissues and testes (when present). In April the ovo-vitelline tissues had a count approx. six times higher than the somatic tissues. In *P. tenuis* the gonads had counts equal to or less than the somatic tissues and the grain density of the ovo-vitelline tissue was only twice that of the somatic tissues. Again these quantitative findings confirmed subjective assessments of isotope distribution in other animals.

DISCUSSION

In most of the triclads we considered, immature, mature and asexual, there were no obvious antero-posterior gradients in the distribution of labelled leucine and this is in contrast to the findings of Coward and Flickinger (1965) on the incorporation of amino acids into the tissues of the asexual, *Dugesia dorotocephala*. They found a peak in uptake at the level of the head and the post-pharyngeal, fission zone of this species. Reasons for this discrepancy are unclear but could be associated with differences either in the species or techniques used. Our results, nevertheless, cast doubt on the general, unequivocal occurrence of antero-posterior gradients in amino acid uptake (possibly reflecting gradients in protein synthesis and general metabolism) in triclads.

The gonads and ovo-vitelline tissues of sexual forms are presumably active sites of protein synthesis and are likely to incorporate high levels of amino acids. This was particularly true of the semelparous *D. lacteum*, where the ovo-vitelline tissues could incorporate six times as much label as somatic tissues. In contrast, the ovo-vitelline tissues of the iteroparous *P. tenuis* incorporated only twice as much label as the somatic tissues and the gonads themselves incorporated amounts equal to or less than those of the somatic tissues. Such a differential uptake supports previous findings that reproductive structures and processes are given more metabolic precedence over somatic structures and processes in semelparous as compared with iteroparous triclads (Woollhead and Calow, 1979).

Table 2. Distribution of grains (number per 900 μm^2) in the somatic and reproductive tissues of triclads

Species	Mean counts ± 2SE Somatic	Ovary	Testis	Ovo-vit.*	F*	df*	P*
D. lacteum (December)†	14.3 ± 2.9 (1)†	25.2 ± 5.6 (1.76)	46.0 ± 5.4 (3.22)	50.8 ± 7.6 (3.55)	36.5	3/116	< 0.05
D. lacteum (April)	10.4 ± 3.3 (1)	27.6 ± 5.1 (2.65)	—	64.0 ± 7.0 (6.15)	87.9	2/89	< 0.05
P. tenuis (March)	18.2 ± 2.8 (1)	12.4 ± 3.3 (0.68)	17.1 ± 4.4 (0.94)	32.7 ± 5.1 (1.80)	19.0	3/116	< 0.05

*F = variance ratio; df = degrees of freedom; P = probability levels; ovo-vit. = ovo-vitelline tissue.
† = Figures in brackets are ratios of counts to somatic counts, dates in brackets indicate when the triclads were collected.

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SUBMANDIBULAR GLAND SECRETORY FUNCTION IN YOUNG ADULT AND AGED RATS

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Abstract—1. An evaluation of submandibular gland secretory function was performed in young adult and aged male and female rats.

2. No significant alterations in submandibular salivary flow rate or the concentrations of total protein, Na^+, K^+ and neutral sugar in the secreted saliva, were observed between different aged animals.

3. Significant sex differences in salivary flow rate and total protein content were found.

INTRODUCTION

There have been many studies reporting morphological changes in human and rat submandibular glands with increased age (e.g. Andrew, 1949; Bogart, 1967; Waterhouse *et al.*, 1973; Scott, 1977). Gland parenchyma is described as being infiltrated by fat and connective tissues and increased numbers of autophagic lipofuscin granules are also observed. With the rat submandibular gland, changes in several descriptive biochemical parameters have also been noted (Bogart, 1970; Kuyatt and Baum, 1981). These include diminutions in RNA, protein, sialic acid and neutral sugar content with glands from aged animals. Furthermore limited age-related functional impairments have also been described. For example, in response to the β-adrenergic agonist isoproterenol, submandibular glands from aged rats show a delayed onset and altered level of both DNA synthesis (Adelman *et al.*, 1972; Roth and Adelman, 1973) and glycoprotein production (Mysliwski and Zurawska-Czupa, 1976).

However we are aware of no studies which examine submandibular salivary secretion *in vivo* among different aged animals. This is surprising since the principal physiologic role of these glands is the exocrine production of saliva, and since submandibular saliva contains many factors critical to oral health (Mandel, 1980). If the descriptive changes in glands noted above were to impair saliva production in older animals, significant alterations in the health and function of the oral cavity would likely result. It was therefore the purpose of the present study to evaluate saliva production from young adult and aged rat submandibular glands. Our results demonstrate, by several measures, that rat submandibular saliva secretion remains generally constant with increased age.

MATERIALS AND METHODS

Male and female Wistar derived rats were used in this study. All animals were obtained from the National Institute on Aging-Gerontology Research Center (Baltimore, MD) colony. These animals have a mean lifespan (50% mortality) of 23–24 months. For this study animals aged 4–7 months were considered "young" adults (6 male, 8 female), and those from 23 to 25 months were termed "aged" adults (6 male, 8 female). Animals were maintained on NIH Purina laboratory chow and water *ad libitum* until used. Room light was provided between 06.00 and 18.00 hr.

All saliva collections were carried out between 09.00 and 12.00 hr. Animals were anesthetized (sodium pentobarbitol 50 mg/kg body wt, intraperitoneally), tracheostomized and the submandibular duct cannulated intraorally as previously described (Bodner *et al.*, 1983a). Secretion was stimulated by pilocarpine HCl (Sigma, 2.5 mg/kg, subcutaneously). The initial drop of saliva was discarded and subsequent flow was collected for 45 min into pre-weighed plastic tubes kept on ice. Following the collection period, saliva was made 1 mM in ethylene diamine tetraacetate to prevent the formation of a calcium-protein precipitate (Dawes, 1981) and stored at -20°C until analyzed. For each analysis, all samples were assayed at one time, and at least in duplicate, to minimize error.

The following parameters were measured with submandibular saliva samples: flow rate, the concentrations of total protein, neutral sugar, Na^+ and K^+. Flow rate was determined gravimetrically assuming the specific gravity of saliva equals 1, and was expressed as μl/min · gland · kg body wt. Total protein was measured by absorbance at 215 nm (Arneberg, 1971), using bovine serum albumin as a standard. Neutral sugar was determined using the hot anthrone procedure of van Handel (1967) using glucose as a standard. Neutral sugar was only measured with samples from male rats due to insufficient sample volumes with females. Na^+ and K^+ were determined by atomic absorption, using a Perkin–Elmer Model 2380 Atomic Absorption Spectrophotometer. Data for neutral sugar content were analyzed for significance by a Student's t-test. Data for all other variables were tested for significance by an analysis of variance.

RESULTS

Submandibular saliva flow rate following stimulation with pilocarpine, did not change ($P > 0.05$) with age (Fig. 1). Different aged rats were equally competent in fluid production. Data for female rats tended to be somewhat less variable, and significantly lower ($P < 0.01$), than flow rate data from male rats.

The two principal cations secreted in mammalian salivas are sodium and potassium. As shown in Figs 2 and 3 there were no age related differences in the

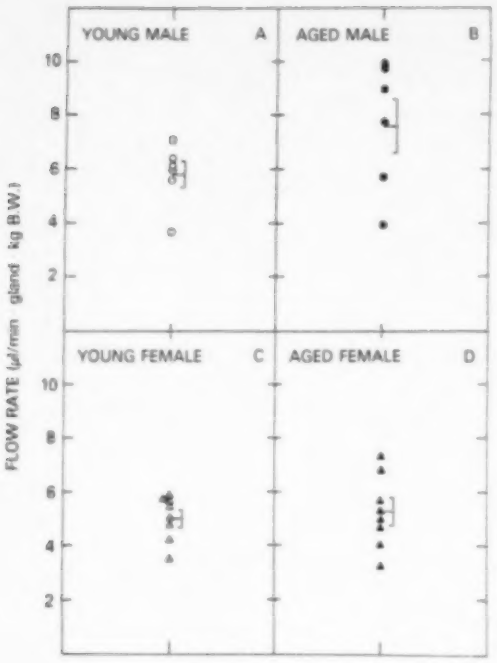

Fig. 1. Flow rate of pilocarpine-stimulated submandibular saliva from different aged male and female rats. Horizontal bars represent the mean ±SEM. Each data point represents results from a single animal and are reported as μl secreted/min · gland · kg body wt (BW).

concentrations of these cations in the samples studied here. All saliva samples evaluated showed comparable levels of potassium (30–40 mM). Most measured sodium concentrations in saliva were also similar, though samples from female rats tended to display higher sodium levels ($P < 0.025$) but also much more variability. It is generally considered that the sodium content of saliva is a reflection of ductal reabsorption of sodium from the isotonic primary fluid while potassium levels mirror the active secretion of the cation by duct cells (Young and Van Lennep, 1979). Based on the results in Figs 2 and 3, it would seem that in the aging rat submandibular gland both these ion transport mechanisms are functionally intact.

Following pilocarpine stimulation most submandibular salivary protein seems derived from acinar, rather than ductal, cells (Abe and Dawes, 1978). Total protein secreted into saliva was similar in young adult and aged rats (Fig. 4). However there was a substantial sex difference noted. Female rat saliva contained significantly more protein (~2.0 mg/ml) than male samples (~1.3 mg/ml, $P < 0.001$). Neutral sugar was measured only in samples from male rats and, as shown in Fig. 5, no age-related differences were observed. Since free glucose levels in saliva are quite low (Mandel, 1980), it seems likely that most of the carbohydrate assayed here is in glycosidic linkage to protein.

DISCUSSION

The rat is a frequently used model of both exocrine secretion (Butcher and Putney, 1980; Baum *et al.*, 1983) and gerontological phenomena (e.g. Goldberg and Roberts, 1981; Gray *et al.*, 1982; Goodrick *et al.*,

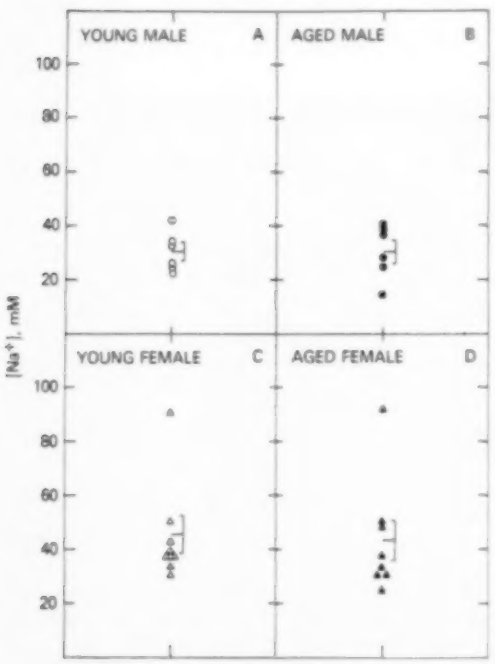

Fig. 2. Sodium (Na^+) concentration in stimulated saliva samples from different aged rats. Samples are those shown in Fig. 1. Horizontal bars represent the mean ± SEM.

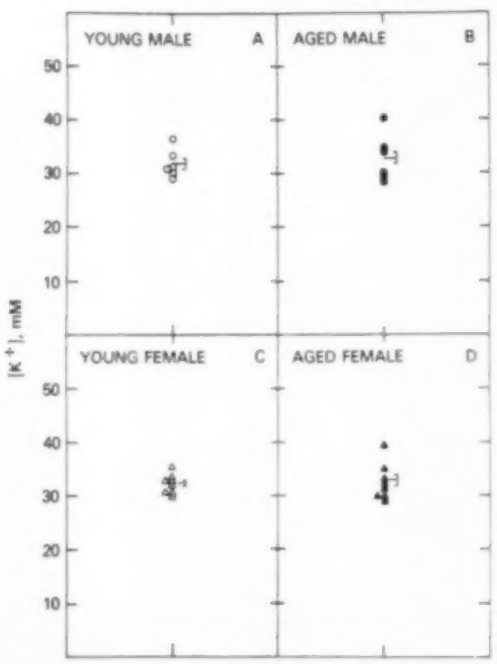

Fig. 3. Potassium (K^+) concentration in stimulated saliva samples from different aged rats. Samples are those shown in Fig. 1. Horizontal bars represent the mean ± SEM.

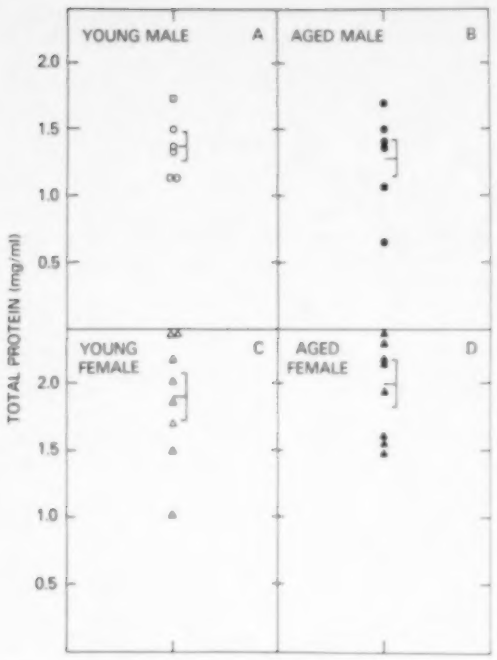

Fig. 4. Protein content of stimulated submandibular saliva samples from different aged rats. Samples are those shown in Fig. 1. Horizontal bars represent the mean ± SEM.

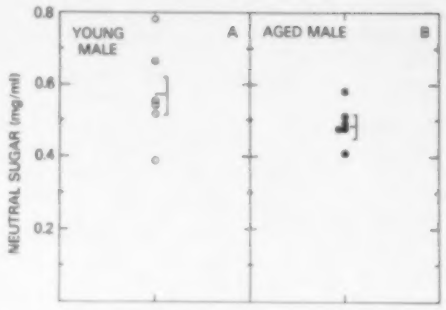

Fig. 5. Neutral sugar content (expressed as glucose equivalents) of stimulated submandibular saliva samples from different aged rats. Samples are those from males shown in Fig. 1. Horizontal bars represent the mean ± SEM.

1983). Also, as noted earlier, both rats and human salivary glands show similar morphological changes with increased age. Yet this is the first study, to our knowledge, to evaluate *in vivo* salivary gland, secretory function with different aged rats. Our results clearly demonstrate that there are no alterations with increased age, in either the amount or composition (with respect to the four variables measured) of rat submandibular saliva obtained after pilocarpine stimulation. Such a finding seems somewhat surprising in view of reported morphological (Bogart, 1967, 1970) and descriptive biochemical (Bogart, 1970; Mysliwski and Zurawska-Czupa, 1976; Kuyatt and Baum, 1981) changes in aging rat submandibular glands. Indeed the latter study was carried out with rats (same sex, age and strain) from the same colony as those used here.

It would appear then that independent of the reported changes in this gland with age, secretory function can proceed "normally" in scenescent rats. Consistent with this interpretation is the recent report for the stimulated human parotid gland, that flow rate (Baum, 1981; Chauncey *et al.*, 1981) and total protein secretion (Baum *et al.*, 1982; Chauncey *et al.*, 1981) do not change with aging. Interestingly structural alterations have also been described in this gland with increased age (Bauer, 1950; Andrew, 1952).

Alternatively it should be noted that stimulation of salivary secretion by pilocarpine (parasympathomimetic) does not provide a complete test of the regulatory mechanisms for submandibular saliva production. For example it has been demonstrated that the volume, protein content, and pattern of proteins of secreted submandibular saliva will change depending on whether pilocarpine, isoproterenol (β-adrenergic agonist) or methoxamine (α-adrenergic agonist) are used as a stimulus (Abe and Dawes, 1978; Martinez and Cassity, 1982). Thus it is possible that pilocarpine may not elicit changes in secretory protein reflective of changes in the glandular soluble protein content as previously reported (Kuyatt and Baum, 1981) whereas, following isoproterenol stimulation, a different result might be seen.

In any event, these data argue strongly against any generalized perturbation in rat submandibular gland function with increased age. A similar conclusion has been drawn from recent studies in our laboratory of rat parotid gland secretory mechanisms (Bodner *et al.*, 1983b). In rat parotid cells both β-adrenergic and muscarinic cholinergic-induced secretory events were stable with age, while α-adrenergic secretion was markedly perturbed. Since the physiological role of saliva is the maintenance and protection of oral tissues involved in the critical functions of food acquisition and digestion, the general preservation of salivary function is of benefit to the host.

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CHITINASE ACTIVITY IN THE CRYSTALLINE STYLE OF THE AMERICAN OYSTER *CRASSOSTREA VIRGINICA*

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Abstract—1. Presence of chitinase in *Crassostrea virginica* crystalline styles was established.
2. Specific activity of chitinase tended to increase towards the anterior portion of the style.
3. Chitinase activity was not stimulated by chitin additions to 1 μm filtered sea water.
4. Kaolin additions to the diet resulted in doubling chitinase specific activity.

INTRODUCTION

There have been few investigations of carbohydrases in bivalve mollusks since the work of Elyakova (1972) and Kristensen (1972). Using an assay which measured the release of *N*-acetylglucosamine (Reissig *et al.*, 1955) from decalcified-deproteinized crab shell chitin, Elyakova (1972) found that only *Modiolus difficilis* out of nine bivalve species produced chitinase with significant activity on this substrate. Enzyme activity on precipitated chitin was more widespread (Elyakova, 1972). It is difficult to ascertain to what extent this apparent difference may have been due to a more favorable enzyme substrate ratio resulting from the two different chitin preparations in that investigation. Elyakova (1972) found chitinases to be weaker and less widespread than cellulases, although Kristensen (1972) demonstrated substantial chitinase activity in representatives of several phyla. In bivalves, chitinase activity was shown to be associated with the digestive gland, and the crystalline style showed only very weak evidence of chitin hydrolysis. Although Wojtowicz (1972) found strong chitobiose activity in the digestive gland of the deep sea scallop, *Placopecten magellanicus*, there was no evidence of this enzyme from the crystalline style and no chitinase activity at all from either source.

In view of the conflicting evidence on the existence and strength of chitinases in aquatic animals there is a clear need to reexamine this group of enzymes and their importance in energy transfer in aquatic systems.

It has been shown that a chitosanase (hydrolyzing deacetylated chitin, chitosan) may also digest cellulose (Price and Storck, 1975) and there is a need for further investigation of enzyme substrate specificity in this group of enzymes. Chitin is an important structural component of many invertebrates, fungi and diatoms (Jeniaux, 1982; McLachlan *et al.*, 1965), and any animal or microorganism capable of utilizing chitin-rich debris will have access to a substantial energy source. Filtering bivalve mollusks rely on suspended organic particles, both living and dead, for their food. Among this material there is likely to be a significant amount of chitin-containing diatoms and structural debris from other sources.

The current study focused on chitinase activity in the crystalline style of the American eastern oyster *Crassostrea virginica*. This species apparently has not previously been studied in detail for chitinase activity. Observations were mainly concerned with enzyme activity in the style and the possibility of enzyme induction according to the nature of the diet.

MATERIALS AND METHODS

Organisms

Oysters (*Crassostrea virginica*) collected from Hog Island (mouth of Patuxent River) and Cornfield Harbor (Potomac River) were maintained in plastic trays suspended from the Chesapeake Biological Laboratory pier in the Patuxent River estuary, Solomons, MD. *Thallassiosira pseudonana* was grown in batch culture and fed on F/2 medium (Guillard, 1975) in diluted seawater (15‰).

Chemicals

^{3}H-acetic anhydride was obtained from New England Nuclear. Tris buffer was from Sigma Chemical Co., St. Louis, MO and all other chemicals used were reagent grade from Baker Chemical Co. or Mallinkrodt.

Experimental procedures

Crystalline styles used for determination of chitinase activity along the style axis were removed from animals in unfiltered sea water. Salinity and temperature remained relatively constant throughout all of these experiments (15‰, 18°C). Seven actively feeding adult oysters were removed from the water and immediately shucked. The styles were removed by lateral incision, approximately mid-way along the style sac. Styles were drained by gently tapping them on a slide, the food tassle (dark mass) was gently removed and the styles were cut into three equal lengths with a scalpel.

Anterior (gut end), mid-, and posterior style sections were pooled from the seven animals. The composite samples were homogenized with a close fitting Teflon-glass homogenizer in 0.05 M Tris buffer, pH 7.61, containing 1% NaCl and 0.02% NaN_3 (TRAZ buffer).

Type of food effects on chitinase activity were determined on randomly selected adult oysters. These animals were scrubbed clean of fouling organisms and depurated for two days in flowing 1 μm filtered sea water. Eight oysters were randomly placed in five separate treatment tanks in a through-flow seawater system. Treatments consisted of un-

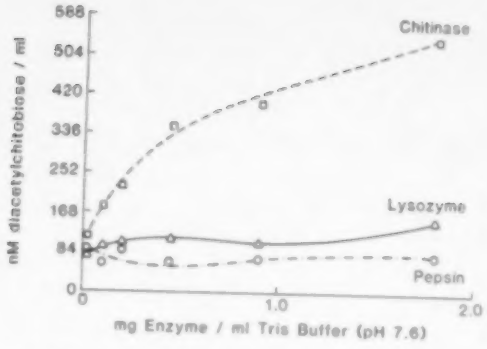

Fig. 1. Effect of hydrolytic enzymes on regenerated chitin. Chitinase (*S. griseus*) and egg white lysozyme activities determined in pH 7.6 TRAZ buffer (see text for details). Pepsin (hog pencreatic) action was determined in 0.01 N HCl.

filtered seawater (15‰), 1 μm filtered seawater, and separate material additions to filtered seawater. The added materials were purified kaolin, purified (regenerated) chitin (see below), and *Thallasiosira* which were added by a diluter system at approximately constant weight density and rates. Crystalline styles were removed from all animals after 72 hr exposure. Animals were removed one at a time from the tanks and styles were immediately removed and maintained in liquid nitrogen until analysis. Each style was thawed, separately homogenized in TRAZ buffer and analyzed for chitinase activity and protein content. In all cases analyses were done on cell-free extracts obtained by centrifugation in a clinical centrifuge.

Chitinase assay

^{3}H-chitin was prepared by reacetylation of chitosan using ^{3}H-acetic anhydride in methanol and aqueous acetic acid (Hirano *et al.*, 1976; Molano *et al.*, 1977). Final specific activity was 398 dpm/nM chitobiose. Chitobiose is the β, 1–4 linked dimer of *N*-acetyl-D-glucosamine (GlcNAc). Each reaction vial contained 168 μg ^{3}H-chitin, and 500 μl enzyme solution in TRAZ buffer. Reactions were terminated with 0.2 ml 10% TCA after 90 min at 30°C. Water soluble hydrolytic release products were collected in 10 ml Aquasol II using a BioRad vacuum manifold. Water insoluble ^{3}H-chitin was retained on Gelman Type A/E glass fiber filters. Each reaction vial was rinsed three times with 0.2 ml distilled water and these aliquots were collected in the Aquasol II.

Enzyme activity was expressed as the release of nM chitobiose per mg protein. Since the styles contain a high concentration of protein these samples were analyzed for protein by the 280 nm/260 nm spectrophotometric method of Warburg and Christian (1942).

RESULTS

Figure 1 illustrates the relative specificity of the assay used to determine chitinase activity. Even lysozyme which is known to hydrolyze chitin has very low activity compared to *S. griseus* chitinase. This is consistent with the binding and hydrolysis kinetics reported by Skujins *et al.* (1973). Their work showed that lysozyme binds and hydrolyzes chitin at a rate three orders of magnitude less than chitinase. While this does not rule out the possibility of a lysozyme in the oyster extract it suggests that disproportionate amounts of the style enzymatic pool would have to be lysozyme in order to account for the chitinase activities observed.

Penicillium islandicum chitosanase requires sequential residues of β 1–4 linked glucosamine and GlcNAc. Extracellular *P. islandicum* chitosanase formed during growth on chitosan failed to hydrolyze the ^{3}H-chitin formed by regeneration. This affirms the specificity of using the regenerated chitin which has greater than 95% of the *N* groups acetylated. Most chitins, as from the blue crab, have from 75 to 90% acetylation depending on method of isolation (Brine and Austin, 1981).

Results in Table 1 apparently represent the first report of chitinase activity in *Crassostrea virginica*. The pooled enzyme preparations from the anterior third (gut end) of the style appear to have higher specific enzyme activity, than the other regions. This suggests that either the chitinase enzyme is produced at higher rates in the anterior region of the style sac or that there is some modulating condition (factor) in the posterior region.

Oysters maintained in flowing 1 μm filtered water continued to produce crystalline styles, thereby indicating some continued feeding activity (Table 2). This is evidenced by the formation of feces during the 72 hr experimental period. Data were obtained only from animals containing styles (3–4 from each treatment).

Styles from unfiltered water had significantly lower chitinase specific activity than did all other treatments. This suggests that substances unique to the sea water particulates induced other enzymes or proteins which in effect diluted the chitinase specific activity.

Kaolin additions to the filtered water resulted in a significant increase in chitinase specific activity. *T. pseudonana* chitin and regenerated chitin treatments

Table 1. Chitinase activity in segments of oyster crystalline styles*

Style region	Protein (mg ml)	nM Chitobiose (ml^{-1})	nM Chitobiose (mg protein^{-1})
Anterior†	0.293‡	1.42	4.85 $\bar{x}$ = 4.89
	0.288	1.42	4.93
Mid-Section	0.454	1.90	4.18 $\bar{x}$ = 4.46
	0.448	2.12	4.73
Posterior	0.602	1.90	3.16 $\bar{x}$ = 3.66
	0.603	2.50	4.15

*Crystalline styles from 7 actively feeding oysters were cut into 3 equal segments. Segments from each of the anterior, central and posterior regions were pooled. Pooled samples were run in duplicate.
†Gut end of crystalline style.
‡Pooled region samples were brought to equal volumes after homogenization.

Table 2. Effect of various feeding treatments on chitinase production in crystalline styles

Treatment	*N*	Mean nM Chitobiose ((mg protein)$^{-1}$ ± SD)
Unfiltered	5	20.2 ± 5.2*
Algae	5	25.0 ± 8.5
Chitin	5	26.5 ± 7.0
1 μm filtered	4	27.5 ± 3.1
Kaolin	6	47.5 ± 22†

*Significantly different from all other treatments at 95% confidence.
†Significantly different from all other treatments at 99% confidence.

resulted in chitinase activity similar to the 1 μm filtered water treatment, indicating no apparent induction of chitinase by these agents.

DISCUSSION

This is apparently the first demonstration of specific hydrolytic activity of chitinase in the oyster. Although there have been reports of chitinase activity in other invertebrates and bivalves (Kristensen, 1972) there have been no identified reports of the enzyme in *Crassostrea virginica*.

Most previous work concerning chitin hydrolysis used chitin substrates which were not of uniform quality and degree of *N*-acetylation (Brine and Austin, 1981). The lack of uniformity in acetylation has definite impact on analytical determinations. These variations are not observed in underived polysaccharides such as laminarin, cellulose, starch and glycogen. However, in the case of chitins the degree of *N*-acetylation dramatically changes polymer chemistry. This in turn may have an important bearing in differential reporting of chitinase activity in different laboratories. It is known that *P. islandicum* produces a chitosanase which hydrolyzes linkage between glucosamine and GlcNAc residues but does not attack back to back GlcNAc residues (Dr D. Eveliegh, Rutgers University, personal communication). Price and Storck (1975) demonstrated a chitosanase from *Streptomyces* (filamentous bacterium) which hydrolyzes β, 1–4 linked glucosamine–glucosamine residues. The significance of using a highly acetylated ^{3}H-chitin (>95%), as is easily obtained by regenerating chitosan (Molano *et al.*, 1977), to determine chitinase activity is obvious. If one uses partially deacetylated chitin (as is most often used), then tissue extracts containing some type of chitosanase could produce end products which may not reflect true chitinase activity. Confirmation of enzyme type would require chromatographic methods. The simplest approach to this problem would be to use a defined chitin substrate so that other enzymatic possibilities are negligible. Chitin regeneration from chitosan has good yields with a high percentage *N*-acetylation (Hirano *et al.*, 1976; Molano *et al.*, 1977). Hydrolytic products from regenerated chitin will likely be from hydrolysis of β, 1–4 linked GlcNAc. Even though this type of chitin is not a "natural" chitin, it is a good substrate for a wide variety of environmental chitinases (Smucker, 1982) and as such should make a good reference substrate.

Kaolin additions resulted in doubling the specific chitinase activity in *C. virginica* (Table 2). Consequences of this are unclear but are consistent with results obtained by Langdon and Siegfried (in press). Using artificial diets to feed *Crassostrea virginica* juveniles, they found improvements in growth rate with additions of kaolin up to 125 mg/l/day. *Mytilus edulis* has improved growth rates when silt is added to its diet (Murken, 1976). The mechanisms of kaolin action are not understood.

The present work shows that chitinase is an integral component of the oyster (*Crassostrea virginica*) digestive system. Chitinase activity was predicted initially, based on the fact that oysters feed on *Thallassiosira* which produce significant amounts of chitin. This food source and detrital as well as colloidal partially degraded chitins are potential food for the oyster.

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EFFECTS OF STARVATION ON THE FINE STRUCTURE OF THE HEPATOPANCREAS IN THE COMMON PRAWN *PALAEMON SERRATUS* (PENNANT)

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Abstract—The ultrastructural changes in the hepatopancreatic cells of the common prawn *Palaemon serratus* (Pennant), induced by starvation were examined. The following observations were made:

1. The fine structure of storage lipid cell (R-cell) and of protein producing cell (F-cell) was mainly affected.
2. Fewer secretory cells (B-cells) were present in the tubule of starved specimens.
3. The absorption mechanism and protein synthesis were disturbed.
4. The most affected organelles were the mitochondria and the rough endoplasmic reticulum.

INTRODUCTION

The liver and pancreas in vertebrate animals are the main sites of both organic and inorganic reserve material (Rothman, 1975). In crustaceans, the hepatopancreas has the function of both the liver and pancreas of vertebrates (Vonk, 1960) and therefore is involved in the secretion of digestive enzymes and the absorption and storage of lipid material (Bunt, 1968; Smith *et al.*, 1975).

Studies concerning the types and function of the cells in the hepatopancreas have been made on crustaceans with emphasis on isopods (Clifford and Witkus, 1971; Schultz, 1973; Smith *et al.*, 1975; Schultz, 1976). Early studies have shown that four types of cells are present in the hepatopancreas of decapods: The embryonic cells (E-cells), the absorptive-storage cells (R-cells), the fibrillar cells (F-cells) and the secretory cells (B-cells) (Jacobs, 1928). Ultrastructural studies established that these cell types exist in Decapoda, although opinions differ regarding their origin (Davis and Burnett 1964; Bunt, 1968; Loizzi, 1971). The latter also depends on animal's state of nutrition (Stanier *et al.*, 1968; Barker, 1979).

Morphological studies in a number of animal groups have related changes caused by starvation with an alteration of the function in organs, such as the liver, kidney or pancreas (Steeves, 1963; Thompson *et al.*, 1974; Davies and King 1977; Rosemark *et al.*, 1980).

The common prawn *Palaemon serratus* (Pennant) is an ecologically important and widely distributed shallow water species which has potential for economic exploitation. It is of interest, therefore, to examine the ultrastructural changes in the hepatopancreas after starvation since hepatopancreas could be a potential indicator organ for early starvation.

MATERIALS AND METHODS

Specimens of *P. serratus* were collected from rock pools at Mumbles Point, Swansea, UK during 1978. All collections were made at low tide and the specimens were then kept in running sea water with 30 ± 1% salinity at ambient temperature for a week before use. After this period samples of five standard-sized individuals were acclimated at 15 ± 1°C in sea water with 30% salinity for 48 hours. The samples were then placed into two crystallizing dishes containing artificial sea water also with 30% salinity and kept for 56 hours. Individuals in the first of these two dishes were fed daily, with chopped fish fingers, throughout the experiment, while specimens in the second dish were starved. The artificial sea water was made up by dissolving "Tropical Marine" salts, distributed by Shirley Aquatics Ltd., Solihull, England, in glass distilled water. After this period, specimens from the two dishes were removed and placed in 5% glutaraldehyde in buffered sodium cacodylate with sucrose added, the hepatopancreas was dissected out and placed in fresh 5% glutaraldehyde solution at pH 7.4 for 1 hr at 0–4°C. The tissues were then washed in several changes of buffered sodium cacodylate with sucrose added followed by post fixation in 1% osmium tetroxide solution for 1 hr at 0–4°C. After dehydration in graded cold acetones the material was embedded in TAAB embedding resin. Sections with gold or silver interference colours were obtained using a Huxley Mark I Ultramicrotome and were mounted on coated copper grids. The specimens were then double stained in 30% uranyl acetate (30 min) followed by lead citrate (10 min) and viewed in a Corinth AEI Electron Microscope.

For histochemical studies, specimens were fixed in Bouin's solution for 24 hours and the hepatopancreas was dissected out. The tissues were then dehydrated in alcohols, cleared in xylene and embedded in paraffin wax. Sections were stained with Sudan Black B, Alcian Blue or Mercury-bromophenol-blue to demonstrate the presence of lipids, carbohydrates or proteins (Pearse, 1968).

RESULTS

There are four cell types in the hepatopancreas of *P. serratus*.

Cell type I (*Fig.* 1.1.)

These cells have few mitochondria containing randomly orientated cristae. The centrally located nucleus is proportionately large compared with the volume of the cell. Proximal to the nucleus there are golgi complexes and amounts of rough endoplasmic reticulum (Fig. 3.1). The cytoplasm possesses abundant

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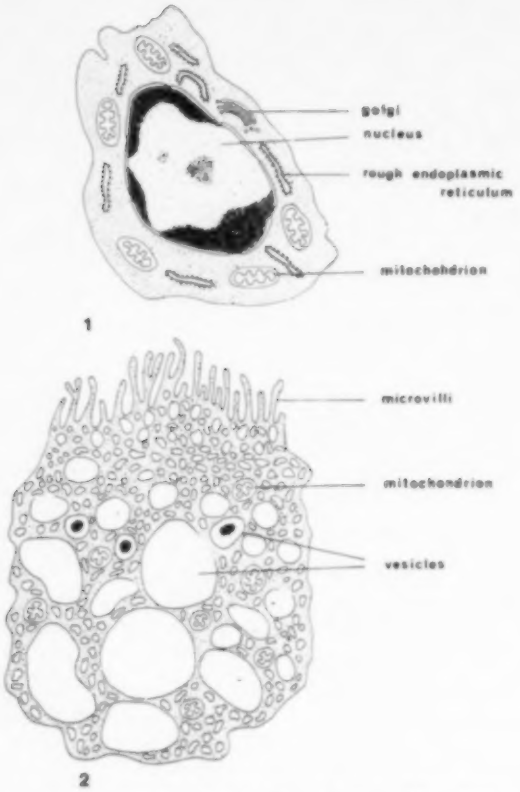

Fig. 1. Diagram showing the fine structure of hepatopancreatic cells of *P. serratus*. (1) Embryonic cell, (2) secretory cell.

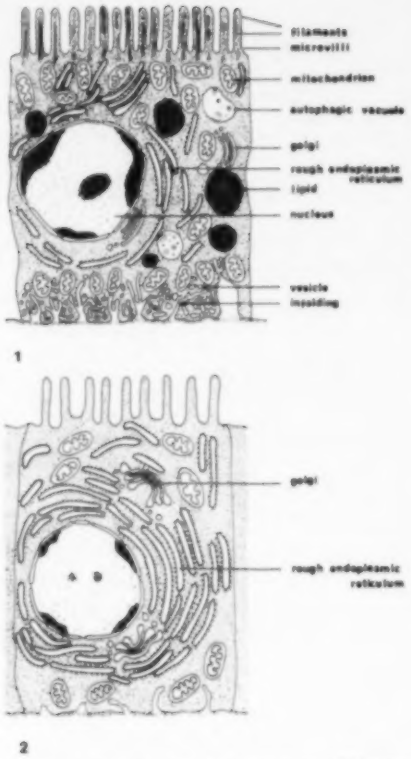

Fig. 2. Diagram showing the fine structure of hepatopancreatic cells of *P. serratus*. (1) Absorptive cell, (2) fibrillar cell.

free ribosomes and vesicles of smooth endoplasmic reticulum. The structure of these cells resembles that of the E-cells of *Carcinus maenas* L. (Stanier *et al.*, 1968).

Cell type II (*Fig.* 2.1)

These cells are characterized by the presence of lipid material (Fig. 3.2). The cytoplasm contains a number of mitochondria, some of which are concentrated below the apical plasma membrane (Fig. 3.2). The nucleus is centrally or basally situated and contains medium electron dense chromatin and one nucleolus (Fig. 3.3). The apical membrane of the cells has microvilli, the surfaces of which are covered by short filaments (Fig. 3.4). The core of each microvillus has filaments which extend deep into the cytoplasm (Fig. 3.4). Rough endoplasmic reticulum and golgi complexes occur throughout the cell (Figs 3.3 and 3.5). In the basal region of the cell the infoldings of the basal membrane are associated with vesicles of the smooth endoplasmic reticulum (Fig. 3.6). Autophagic vacuoles containing material of different configurations with low electron density are also present in the cytoplasm (Fig. 3.5). These cells are similar to those described as R-cells in the crayfish hepatopancreas (Loizzi, 1971).

Cell type III (*Fig.* 2.2)

These cells contain abundant rough endoplasmic reticulum, the cisternae of which are filled with a

Fig. 3.1. Electron micrograph showing an embryonic cell (E). Note the small number of mitochondria (m). Nucleus (n), Golgi complex (gc). Scale bar 1 μm.

Fig. 3.2. Electron micrograph showing two absorptive cells (R). Note the mitochondria (arrows) beneath the microvilli (mi) and the large amounts of lipids (l). Scale bar 3 μm.

Fig. 3.3 Electron micrograph showing an absorptive cell (R). Note the abundant rough endoplasmic reticulum (rer) and the large lipid droplets (l). Nucleus (n). Scale bar 4 μm.

Fig. 3.4. Electron micrograph showing the microvilli (mi) of an absorptive cell. Note the filaments on the surface of the microvilli (arrowheads) and the filaments in the core of each microvillus (arrow). Scale bar 0.3 μm.

Fig. 3.5. Electron micrograph showing the apical region of an absorptive cell. Note the presence of autophagic vacuole (av) proximal to microvilli (mi). Lipid (l), Golgi complex (gc). Scale bar 2.5 μm.

Fig. 3.6. Electron micrograph showing the basal region of an absorptive cell. Note the infoldings of the basement membrane (i) associated with vesicles (arrows). Mitochondria (m), rough endoplasmic reticulum (rer). Scale bar 1 μm.

flocculent material (Fig. 4.1). Golgi complexes are present in the cytoplasm, the active face of which produce vesicles (Fig. 4.2). The cytoplasm contains abundant free ribosomes but fewer mitochondria than in cell type II (R-cells). The structure of these cells resembles that of the F-cells in the crayfish hepatopancreas (Loizzi, 1971).

Cell type IV (*Fig.* 1.2)

This type of cell is characterized by the large number of vesicles, which initially occupy a considerable part of the cell and eventually occlude it (Fig. 4.3). The apical membrane has an undulated appearance and the microvilli become more pronounced than in either II-cell type or III-cell type (Fig. 4.3). These cells contain

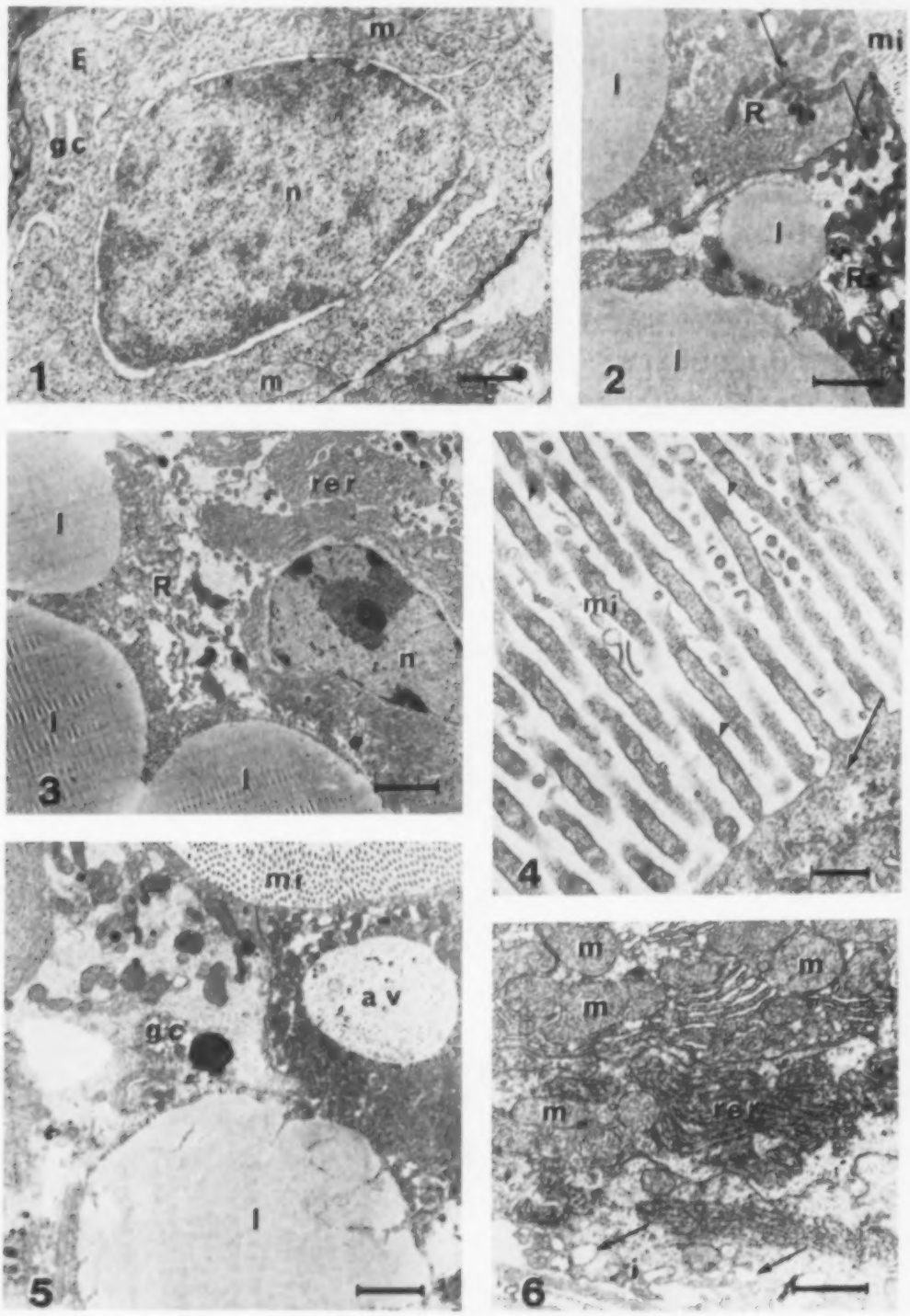

Fig. 3.

a small number of mitochondria and different sized vesicles with electron dense material (Fig. 4.4). The latter have similar structure to those described in the hepatopancreas of the crabs as deposits of calcium (Stanier *et al.*, 1968; Negrel, 1981). These vesicles are surrounded by complete or incomplete membranes (Fig. 4.4). These cells are the secretory cells and resemble the B-cells of Davis and Burnett (1964) and Stanier *et al.* (1968).

After 56 hr starvation the E-cells and the B-cells in the hepatopancreas of *P. serratus* were unchanged from those in fed specimens, but there are fewer secretory cells in the tubule. The cell types which were affected were the R-cells (II) and the F-cells (IIII).

(a) *Cell type* II (*R-cells*) (*Fig.* 5.1)

In the apical region of these cells the cisternae of the rough endoplasmic reticulum became shorter (Fig. 6.1) compared with those in fed specimens. The mitochondria became enlarged and contained fewer cristae (Fig. 6.1). Smaller and spherical mitochondria, however, occur at the basal region of these cells. In the central

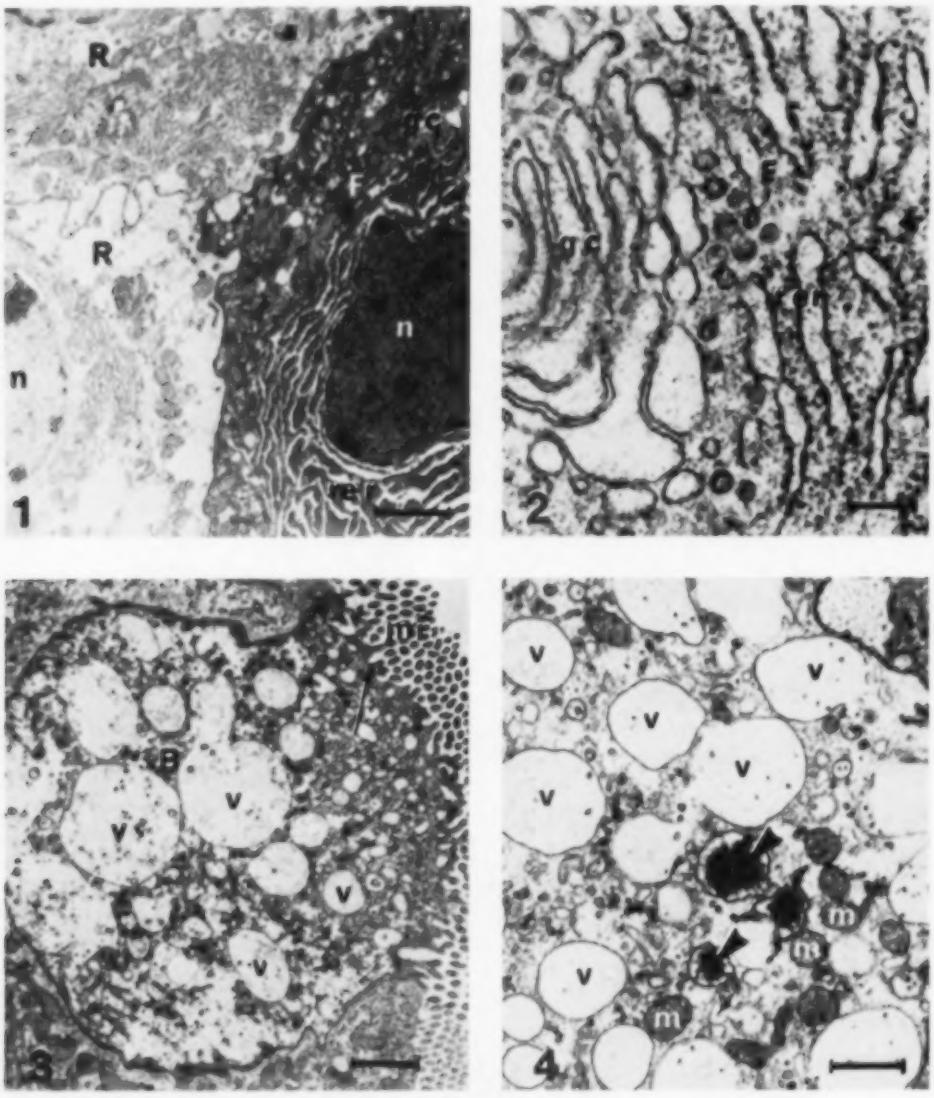

Fig. 4.1. Electron micrograph showing a fibrillar cell (F) and two absorptive cells (R). Note the abundant rough endoplasmic reticulum (rer) in the F-cell. Nucleus (n), Golgi complex (gc). Scale bar 2 μm.

Fig. 4.2. Electron micrograph showing active Golgi complex (gc) in association with rough endoplasmic reticulum (rer) in a fibrillar cell (F). Scale bar 0.2 μm.

Fig. 4.3. Electron micrograph showing a secretory cell (B). Note the numerous vesicles (v) within the cell and the undulating appearance of the apical membrane (arrow). Microvilli (mi). Scale bar 1.4 μm.

Fig. 4.4 Electron micrograph showing cytoplasm of a secretory cell. Note the numerous vesicles (v) some of which contain a dielectronic material (arrowheads) and the small spherical mitochondria (m). Scale bar 0.8 μm.

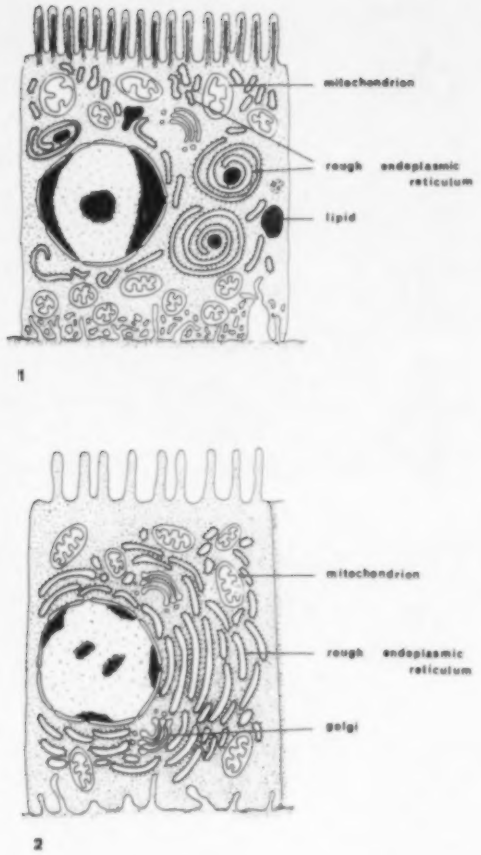

Fig. 5. Diagram showing the fine structure of hepatopancreatic cells of *P. serratus* after a period of starvation. (1) Absorptive cell, (2) fibrillar cell.

and basal region of the cell the rough endoplasmic reticulum has been partially transformed into multilayered whorls (Fig. 6.4). The remaining rough endoplasmic reticulum consists of straight, isolated short cisternae (Fig. 6.2). Electron dense inclusions which are believed to be lipid droplets, are present throughout the cytoplasm, sometimes occuring in the centre of the rough endoplasmic reticulum whorls (Fig. 6.4). This is supported by the histochemical studies at the light microscope level.

(b) *Cell type* III (*F-cells*) (*Fig.* 5.2)

Differences were observed in the rough endoplasmic reticulum and the mitochondria of these cells in starved specimens. The rough endoplasmic reticulum consisted of short cisternae, which were dilated in places (Figs 6.5 and 6.6). Compared with fed specimens there were fewer but enlarged mitochondria (Fig. 6.6).

DISCUSSION

It has been established that the hepatopancreas in crustaceans consists of different cell types, which have functional differences (Davis and Burnett, 1964; Barker, 1979). In the hepatopancreas of *P. serratus* there are four types of cells which have similar ultrastructural characteristics to those described, as E-cells (embryonic), R-cells (absorptive), F-cells (fibrillar) and B-cells (secretory), in other crustaceans by many investigators (Van Weel, 1955; Davis and Burnett, 1964; Bunt, 1968; Barker, 1979).

The F-cells manufacture the digestive enzymes (Van Weel, 1970; Loizzi, 1971) and in *P. serratus* are probably derived from the E-cells. This is supported by the increased amount of rough endoplasmic reticulum in later stages of E-cell development. Similar suggestions have been made by Bunt (1968) and Stanier *et al.* (1968) for the hepatopancreas of other crustaceans. The F-cells may become secretory cells, secreting their products into the lumen. This could agree with observations made by Stanier *et al.* (1968) and Loizzi (1971) working on *C. maenas* and *Procambarus clarkii* (Girard). They postulated that F-cells become B-cells. In contrast to this Davis and Burnett (1964), reported that in the hepatopancreas of *Procambarus blandinqii acutus* (Girard) the F-cells are derived directly from the B-cells by a gradual process of change.

The R-cells are generally characterized by considerable number of lipid inclusions and are thought to be derived independently from E-cells as in the hepatopancreas of other crustaceans (Stanier *et al.*, 1968; Loizzi, 1971). They function in a manner similar to the vertebrate food absorptive cells (Ito, 1965; Negrel, 1981).

After 56 hr of starvation the main differences in both R-cells and F-cells were the enlargement of mitochondria and the appearance of the rough endoplasmic reticulum. Similar changes in mitochondria and rough endoplasmic reticulum have been reported in the midgut of starved *Aëdes aegypti* L. (Bertram and Bird, 1961) and in the venom system and acid gland of starved *Nasonia vitripennis* (Walker) (Insecta: Hymenoptera) (Ratcliffe and King, 1969, 1970). The changes which occur in the R-cells after starvation may be reflected in the function of these cells. The changes in the apical region of the cells, which is involved in absorption (Loizzi, 1971), and the concentric form of the rough endoplasmic reticulum, suggest that hypoactivity exists in these cells. This is also supported by the reduction of cristae in mitochondria in this region. The explanation of the whorls observed in the endoplasmic reticulum is unknown. Ratcliffe and King (1970) described similar rough endoplasmic whorls in the venom system of starved *N. vitripennis*, correlated perhaps with a lack of amino acid incorporation and protein synthesis. In *P. serratus*, however, since lipid material was present in the centre of the whorl, it is possible that these inclusions were associated with the synthesis and store of lipid material, as an initial modification in fasting.

The rough endoplasmic reticulum changes observed in the F-cells after 56 hr starvation may be related to protein synthesis. This is supported by Ekholm *et al.* (1962), who postulated that the susceptibility of endoplasmic reticulum to physiological changes results from its rapid turnover of structural protein and amino-acids during protein synthesis.

Thus starvation of *P. serratus* for 56 hr mainly affects the absorptive and fibrillar cells in the hepatopancreas, where the absorption mechanism and protein synthesis are disturbed.

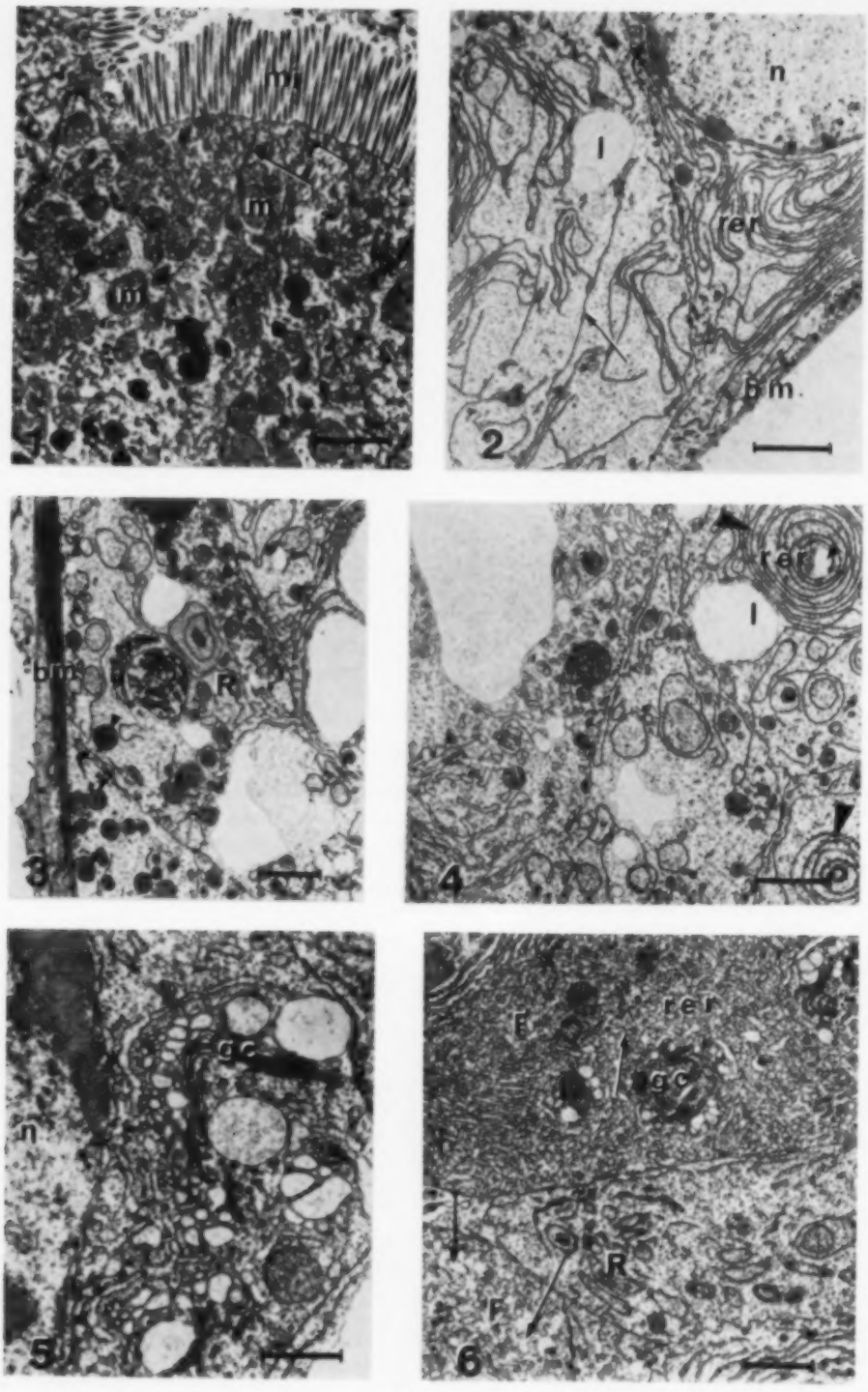

Fig. 6.

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Fig. 6.1. Electronmicrograph showing the apical region of an absorptive cell after starvation. Note the appearance of the mitochondria (m) and the filaments in the core of the microvilli (mi) (arrow). Scale bar 2 μm.

Fig. 6.2. Electron micrograph showing the basal region of an absorptive cell after starvation. Note the appearance of the rough endoplasmic reticulum (rer) near the basal membrane (bm), some cisternae of which are isolated (arrow). Nucleus (n), lipid (l). Scale bar 3 μm.

Fig. 6.3. Electron micrograph showing the basal region of an absorptive cell (R) after starvation. Note the small spherical mitochondria (arrowheads) proximal to basal membrane (bm). Scale bar 3 μm.

Fig. 6.4. Electron micrograph showing an absorptive cell after starvation. Note the concentric whorls of the rough endoplasmic reticulum (big arrowheads), and the presence of lipid material in the centre of the whorl (small arrowhead). Lipid (l), rough endoplasmic reticulum (rer). Scale bar 3 μm.

Fig. 6.5. Electron micrograph showing golgi complex (gc) in fibrillar cells of starved specimens. Nucleus (n). Scale bar 1 μm.

Fig. 6.6. Electron micrograph showing fibrillar cells (F) of starved specimens. Note the swollen cisternae of the rough endoplasmic reticulum (arrows). Golgi complex (gc), rough endoplasmic reticulum (rer). Absorptive cell (R). Scale bar 2.5 μm.

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EXPERIMENTAL AND FIELD RECORD OF PREFERRED TEMPERATURE IN THE NEOTROPICAL AMPHISBAENID *AMPHISBAENA MERTENSI* STAUCH (REPTILIA, AMPHISBAENIDAE)*

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Abstract—1. Temperature preference in experimental thermal gradient in the laboratory was determined and field preferred temperature was recorded, for *Amphisbaena mertensi*.

2. Mean preferred temperature in 52 experiments averaged 21.4°C which was close to those recorded in 16 specimens measured in the field (21.1°C).

3. Selected temperature varied from 12 to 27°C in the laboratory, and data taken in the field ranged from 18 to 26°C.

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INTRODUCTION

Amphisbaenians are limbless Squamata highly adapted to subterranean life, which presumably separated from lizard stock during the Jurassic (Carrol and Galton, 1977). Judging from the fossil record, the fossorial habit of the group might have taken place a long time ago, since well differentiated forms are known as far back as the Miocene (Gans, 1969; Romer, 1966). Thus, amphisbaenians may share some common adaptations with more recently fossorial lizards and snakes, but can have special features due to the long subterranean way of life.

Some amphisbaenians have striking physiological characteristics of blood and muscles that are different from other reptiles (Johansen *et al.*, 1980; Weber *et al.*, 1981). Whether these features are characteristic of amphisbaenians as a group is an open question. The geographic distribution of amphisbaenians, restricted to tropical and subtropical areas (Gans, 1968), and other evidences such as the influence of minimum temperature on the number of body annuli (Vanzolini, 1968) suggest low tolerance of amphisbaenians to cold. However, no data on thermal parameters in amphisbaenians are available except for a study of *Trogonophis wiegmanni* from north Africa (Gatten and McClung, 1981).

This investigation concerns thermal selectivity in *Amphisbaena mertensi*, a species widely distributed throughout southern Brazil (Gans, 1966), and rather common in some areas of the State of São Paulo.

MATERIAL AND METHODS

Amphisbaena mertensi was collected in ploughed fields near Rio Claro (22°25′S; 47°32′W), State of São Paulo. Soil temperature was recorded during collecting, at the depth the animals were found. The amphisbaenas were kept in terraria, and fed on a diet of termites, mealworms and raw meat. Terraria were maintained at 25°C and approximately 12:12 photoperiods, and the soil was kept moist. Amphisbaenas were in good health, and most of them gained weight in captivity. Animals were not fed for 7 days prior to experiments.

Thermal gradient chambers, measuring 34 × 8 × 70 cm (l × w × h) and filled with soil from collecting sites, were constructed of wood with glass side windows. The bottom of each chamber was provided with a coiled plastic tube internally, which could be connected to a circulating thermostatic bath. Thermometers placed at different positions inside the chamber allowed readings without disturbing the animals.

In each experiment amphisbaenas were placed in individual chambers, and the glass side windows covered with dark cardboard. The boxes were kept in a room at 25°C for 24 h, and animal position was recorded every 8 h. After this previous adaptation of each amphisbaena to the chamber, the area with coiled tube was covered with styrofoam slabs and moved to a room at 32°C. Animal positions were recorded every 8 h for 24 h. The experiments were conducted with the chamber in vertical and horizontal positions, and each case considered separately. In all 21 amphisbaenas weighing 9.3 to 31.2 g (mean 20.2 g) were used.

RESULTS

Amphisbaenas kept at 25°C previous to the settling of the thermal gradient, dug extensive gallery systems through the chamber, but had a tendency to remain close to the bottom most of the time. After the gradient had settled, amphisbaenas could be watched through the window by removing the cardboard so that disturbance was minimum. Mean preferred temperature, mean minimum voluntary, mean maximum voluntary temperatures, and field temperatures recorded at collecting are shown in Table 1. As there was no significant difference ($P > 0.05$) between the vertical and horizontal gradient, these data are grouped. Separation in Figs. 1 and 2 is only for graphical purposes. In spite of the fact that selected temperatures

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Table 1. Mean preferred temperature (MPT), mean minimum voluntary temperature (MMV), mean maximum voluntary temperature (MV), field temperature (FT) and weight

	n	$\bar{x}$	SD	Range
MPT (°C)	52	21.4	2.7	15.5–26.0
MMV (°C)	52	19.7	4.4	12.0–26.0
MV (°C)	52	23.1	4.8	17.0–27.0
FT (°C)	16	21.1	2.6	18.0–26.0
Weight (g)	21	20.2	7.2	9.3–31.2

ranged from 12 to 27°C, almost 80% of the animals remained between 16 to 25°C (Fig. 2). Experimental data on mean preferred temperature were not significantly different ($P > 0.05$) from the mean temperature at collecting sites, recorded between 15 and 27 cm depth.

DISCUSSION

As fossorial reptiles, amphisbaenians are typical thigmothermic animals in the sense of Cowles (1940). However, as suggested by Brattstrom (1965) for subterranean lizards and by field observations on the amphisbaenid *Agamodon anguliceps* (Gans, 1968), they may select a given temperature by following the thermal gradient of the soil. It seems that there is a tendency toward a low preferred temperature for subterranean Squamata, judging from the data reported for burrowing snakes of the family Uropeltidae (Gans, 1973), and for the amphisbaenian *Trogonophis wiegmanni* (Gatten and McClung, 1981). The same holds for *A. mertensi*, in which the mean preferred temperature of 21.4°C is quite low for Squamata of tropical regions (Brattstrom, 1965). This value is very close to that reported for *T. wiegmanni*, whose preferred temperature varied from 21.7 to 23.4°C according to time of day (Gatten and McClung, 1981). *A. mertensi* has no tendency towards daily variation and showed a wider preferred temperature range than *T. wiegmanni*. As acclimation for preferred temperature may be complete within two days (Bennet and Dawson, 1976) it is unlikely that the lack of daily variation can be attributable for keeping *A. mertensi* at 25°C and *T. wiegmanni* at 22°C. These values of preferred temperature in *T. wiegmanni* and *A. mertensi* are very close to those reported for subterranean lizards and snakes (Miller, 1944; Gans, 1973). The range of preferred temperature in *A. mertensi* is also close to 15–25°C, as reported for lizards of the genus *Anniela* (Miller, 1944). Most of the data on *A. mertensi* range from 16 to 25°C (Fig. 2), which is in good agreement with data taken in the field. Stewart (1965) also found that mean body temperature in an experimental gradient corresponded with those taken from natural populations in two species of snakes of genus *Thamnophis*. At first approach these data may suggest that amphisbaenians have no special characteristics in regard to preferred temperatures as compared with other subterranean Squamata. Further studies based on more species of amphisbaenids from different habitats will be needed to support this hypothesis.

Ecological information on *A. mertensi* is scant and most data must be inferred from scattered reports or indirect evidences (Vanzolini, 1948). Some of the *A. mertensi* used in this study were collected during ploughing activities at a depth between 15 and 27 cm, in the morning. Temperature at that depth varied between 18 and 19°C. Injured animals were dissected, and full stomach contents revealed that the amphisbaenas had eaten recently. Reptiles of differing groups are known to have thermophilic responses after feeding (Regal, 1966; Gatten, 1974), and the fact that *A. mertensi* can tolerate temperatures around 18°C after feeding, reinforces the data on low thermal preference. Soil temperatures in open areas such as the ones where *A. mertensi* was collected may have a maximum daily range of 40°C at surface level but a variation of only 1.1°C at 30 cm depth in sandy soil (Seltzer, 1942). Not only temperature but humidity as well are not much affected below 30 cm even in unprotected soil (Camargo, 1963). Soil moisture appears to be more critical than temperature in determining amphisbaenian distribution as suggested by Gans (1968). Thus, during the cold and dry season *A. mertensi* goes deeper in dry sites but remains close to the surface in wet sites. For a predator like an amphisbaenian it is likely to be advantageous to have a wide range of preferred temperatures, as it increases the habitat utilization. In fact, stomach contents reveal many

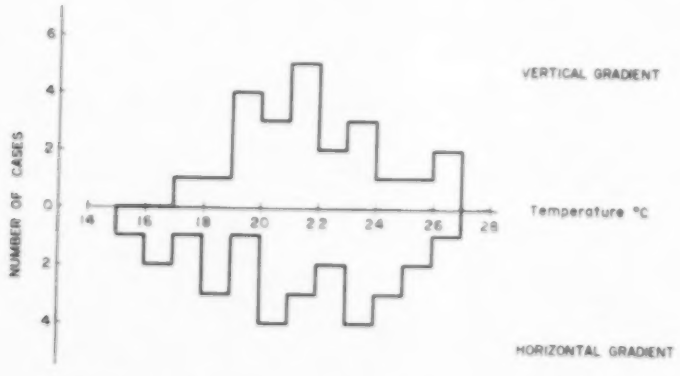

Fig. 1. Mean preferred temperature in vertical and horizontal gradient.

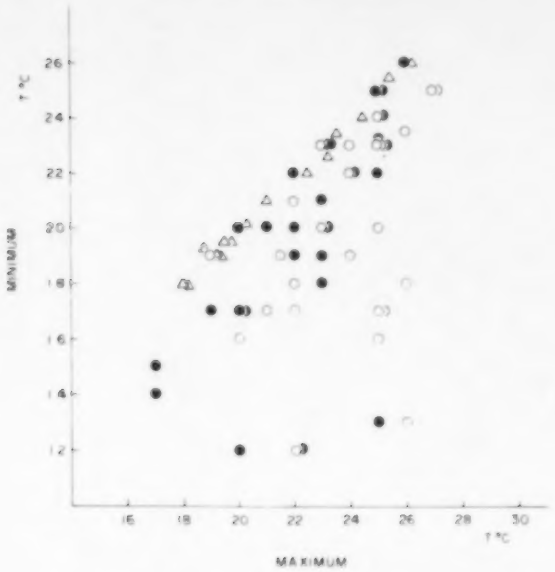

Fig. 2. Preferred temperature range. Coordinates represent the maximum and minimum values observed in each experiment. Open circles—vertical thermal gradient; solid circles—horizontal thermal gradient; triangles—temperature taken in the field.

invertebrate items that cannot be found in a thin shallow soil layer (Lopes and Abe, in preparation).

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OXYGEN TRANSPORT PROPERTIES IN THE STARLING, *STURNUS VULGARIS* L.*

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Abstract—1. Several hematological parameters related to respiratory function of blood (blood volume, hemoglobin, hematocrit, erythrocyte number, P_{50}, Bohr effect, organic phosphates) plus the myoglobin concentrations of different muscles were analyzed in starlings (*Sturnus vulgaris* L.) during their fall migration and winter stay in north-east Spain (Delta del Ebro, Tarragona).

2. The classical hematological values were: Hc = 45.2–47.3%; Hb = 14.2–16.3 g%; erythrocyte number = 4.42–4.59 · 10^6; reticulocytes = 2.6–5.7%; MCM = 29.7–34.2 $\mu\mu$g; MCHC = 30.1–34.4%; MCV = 100.7–110.9 μm^3. No trend seemed evident in these values in relation to the migratory process.

3. The mean blood volume was 16.95 ± 2.5 ml/100 g b.w., and the erythrocyte volume was 6.95 ± 1.41 ml/100 g b.w. The P_{50} was 38.1 ± 4.56 mmHg and the Bohr effect—0.472 ± 0.04; the oxygen capacity was 19.84 ± 3.35 ml O_2 %.

4. The intraerythrocytic phosphates levels were: inositol pentaphosphate (IPP) = 3.47–4.10 μmol/ml RBC; ATP = 5.57–5.90 μmol/ml RBC; and the other phosphates were 2.15–2.66 μmol/ml RBC.

5. The myoglobin concentration ranged from 0.68 mg Mb/g wet muscle in the leg to 7.2 mg Mb/g in the heart or 9.5 mg Mb/g in the pectoral muscle. Significantly high values were found in the birds on their arrival from migration when compared to the specimens resting in the winter ground.

6. The organic phosphates in the red blood cells presented uniform distributions in the different lots analyzed. However, the molar relationship between the phosphate compounds and the hemoglobin showed a tendency to increase during the winter stay.

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INTRODUCTION

In earlier papers we have analyzed respiratory properties in several wild passeriformes (Palomeque *et al.*, 1980a), in apodiforms (Palomeque *et al.*, 1980b) and in urban and homing pigeons (Palomeque and Planas, 1978; Riera *et al.*, 1983). Our intention was to look for adaptations of the oxygen transport capacity to the characteristically high metabolic rates and peculiar biology and flying habits (Berger and Hart, 1974; Hails, 1979) of these species. Following this line, the starling (*Sturnus vulgaris* L.) offered us a double interest, being a powerful flapping flyer and a migratory bird from our country.

This species of starling was recently studied for the energy expenditure in breeding and in nestling chicks (Biebach, 1979, 1981; Clark, 1982) and the seasonal variations of protein, fat and iron in liver (Osborn, 1979) were analyzed. In blood data, for the same species, we found the reference of Kostelecka-Myrcha *et al.* (1972) on hematological changes (hematocrit, hemoglobin content and erythrocyte number, and size) in nestling starlings during the first 21 days of life, as well as, a study on the permeability of its erythrocytes (Hunter and Lim, 1978).

We thought that determinations done at different periods of migration could possibly show some adaptive changes. In the present paper, we have analyzed several blood parameters related to the respiratory function (hematological data, blood volume, oxygen affinity, intraerythrocytic organophosphates) and the myoglobin concentration in different muscles, at different stages of their stay in winter grounds.

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†To whom all correspondence should be addressed.

MATERIAL AND METHODS

The starlings (*Sturnus vulgaris* L.) were caught in the Delta del Ebro (Tarragona, Spain) with japanese nets, during their winter migration. The different lots analyzed, according to the date of capture, were: (A) 16 November 1980; (B) 29 January 1981; (C) 2 November 1981; (D) 15 December 1981. In this region, the starlings arrive from central Europe in October and the return migration takes place in February. Therefore we considered these lots of capture to represent a post-migration state, a resident condition and a pre-migratior period.

After capture, the starlings were transported to our laboratory, where they were immediately bled to death by heart puncture, with a heparinized needle (30/8 gauge) attached to a catheter, the end of which was introduced into a plastic tube. A vibration device was used to facilitate the blood flow and avoid coagulation of the sample. The birds were first weighed alive, using a Ohaus balance, and again after death. The wing surface was determined by planimetry and the wing index was calculated according to Berger and Hart (1974).

The hematocrit was determined in microhematocrit capillary tubes and centrifuged at 11000 rpm for 6 min in a Hemofuge, Heraeus (Germany). The hemoglobin concentration was measured with Drabkin's reagent (Drabkin and Austin, 1935) in 0.01 ml of blood, and the standards were taken from Hycel. The erythrocyte number was determined using a Thoma-Zeiss counting chamber, and the reticulocytes were identified with a cresyl brilliant blue stain (Lucas and Jamroz, 1961). From the above data the hematimetric indexes were calculated.

The intraerythrocytary organic phosphates were extracted with 10% trichloroacetic acid from 0.5 to 1 ml of RBC, having been previously washed three times in a phosphate-free saline solution. The main organic phosphates in the extracts were determined according to the following methods: (a) ATP—Bücher's method (1947), using a Boehringer-Mannheim kit (Germany); (b) Inositol polyphosphate (IPP)—technique of Oshima *et al.* (1964) with some modifications (Riera *et al.*, 1983); and (c) inorganic phosphate (P_i) technique of Ames and Dubin (1960).

The blood volume was determined by intracardiac injection of Blue Evans, adapting the technique of Newel and Schaffer (1950) described for chickens.

The oxygen affinity of the blood (P_{50}) and the Bohr effect were determined by the mixing method (Lenfant and Johansen, 1965), as described earlier (Hughes *et al.*, 1975), using gas mixtures with various proportions of CO_2. The analysis were carried out in an Astrup, BMS 3 model, Radiometer (Copenhagen, Denmark); the polarographic electrodes were calibrated with standard gases and precision buffers. All the analysis and calibrations were done at 41°C and all values referred to pH = 7.5.

The oxygen capacity of the blood was measured according to Tucker's method (1967).

The myoglobin concentrations in different muscles (heart, pectoral, wing, tight and gastrocnemius) were determined using the De Duve method (1948) modified by Anthony *et al.* (1959). The optical densities at 540, 568, 575.5 and 583.8 nm of filtered extracts, saturated in CO, were measured in a Spectronic 2000 (Bausch and Lomb). The weights of the whole organs or muscles were determined in a Mettler H 20 T balance.

A non-parametric analysis, done according to the distribution-free test of Kruskal-Wallis, was used for the myoglobin and phosphates data. In the indicated cases a Mann–Whitney U test or Student *t*-test was applied. The distribution-free test, for ordered alternatives of Jonckeere and Terpstra, was also applied to the inorganic phosphates (P_i) data in Table 2.

RESULTS

The body weights, wing indexes and hematological values from starlings, caught in different captures, are shown in Table 1. No statistical differences were evident between date of capture and anatomical and hematological values.

The red blood cell organic phosphates of the starling (Table 2), showed uniform distribution of IPP and ATP fractions but not in P_i ($P < 0.01$). At the same time, no sexual differences were appreciated in this bird population. The molar relationship between the phosphate compound and the hemoglobin had a tendency to increase in the specimens captured in the middle of December or in January, more than in birds that had just arrived (lots A and C), where the molar ratio was lower; there were no significant sexual differences.

In the Table 3, the blood volume and the oxygen transport capacity of the blood are shown. The average blood volume was 16.95 ± 2.25 ml/100 g b.w., and the erythrocyte volume 6.95 ± 1.4 ml/100 g b.w. In a group of 10 specimens, in which the sex was previously determined, no statistically significant sexual differences appeared. In this table, the mean value of P_{50} and Bohr effect are also included. The oxygen capacity of blood (19.84 ml O_2/100 ml) represented 1.299 ml O_2/g Hb, which was very close to the theoretical limit.

The myoglobin concentrations in several muscles of free living starlings, captured in or around the arrival

Table 1. Body weights, wing indexes and hematological values in different groups of starlings (mean ± SE)

Group	Date of capture	Body weight (g)	Wing index $W^{2.3}/A$* ($g^{2.3}$ cm^2)	Hematological values						
				Hc (%)	Hb ($g\%$)	RBC count (10^6)	Retycul (‰)	MCV (μm^3)	MCHb ($\mu\mu g$)	MCHbC (%)
A	18 Nov. 1980	64.9 ± 5.6 (6)	0.065 (6)	45.4 ± 2.2 (4)	15.3 ± 0.7 (4)	4.51 ± 0.18 (4)	5.7 ± 0.6 (4)	100.7 ± 3.9 (4)	34.1 ± 0.5 (4)	33.7 ± 1.7 (6)
B	29 Jan. 1981	65.1 ± 4.2 (17)	0.076 (12)	45.2 ± 3.6 (17)	14.2 ± 1.5 (6)	4.42 ± 0.77 (9)	2.6 ± 0.5 (5)	108.9 ± 14.9 (9)	29.7 ± 4.6 (9)	30.1 ± 1.7 (6)
C	10 Nov. 1981	66.6 ± 3.6 (29)	0.081 (14)	47.3 ± 2.9 (32)	16.2 ± 1.4 (32)	4.59 ± 0.53 (13)	4.7 ± 1.1 (10)	110.9 ± 15.1 (14)	33.6 ± 1.8 (14)	34.4 ± 3.3 (32)
D	15 Dec. 1981	64.6 ± 4.6 (18)	0.068 (17)	45.2 ± 3.0 (18)	14.4 ± 1.2 (18)	4.43 ± 0.28 (6)	4.1 ± 1.7 (5)	104.5 ± 7.9 (6)	34.2 ± 2.9 (6)	31.8 ± 1.5 (18)

In parentheses, the number of specimens.
*W = body weight; A = wing surface.

Table 2. Values of the main intraerythrocitary organic phosphates in the four lots of starlings, according to the date of capture and differentiated by sex (mean ± SE)

Group	Date of capture	Number of specimens	IPP (μmol/ml RBC)	ATP (μmol/ml RBC)	P_i (μmol/ml RBC)	P total (μmol/ml RBC)	%	IPP	ATP (μmol/μmol Hb)	IPP + ATP	Hb (μmol Hb/ml RBC)
A	18 Nov.	5	4.10 ± 0.2	5.57 ± 0.27	2.66 ± 0.11	41.80 ± 2.42	90.96 ± 2.98	—	—	—	—
B	29 Jan.	5	3.84 ± 0.09	5.87 ± 0.40	2.15 ± 0.13	43.06 ± 1.95	91.14 ± 1.40	0.78*	1.24*	2.02*	4.74*
C	2 Nov.	4	3.75 ± 0.13	5.61 ± 0.18	2.41 ± 0.09	41.95 ± 1.30	90.50 ± 0.96	0.73†	1.05†	1.78†	5.34†
D	15 Dec.	4	3.47 ± 0.20	5.90 ± 0.13	2.25 ± 0.18	39.65 ± 2.67	92.60 ± 2.73	—	—	—	—
Sex											
Males		6	3.88 ± 0.24	5.78 ± 0.32	2.41 ± 0.24	42.53 ± 2.23	90.9 ± 1.5	0.74	1.10	1.84	5.24
Females		11	3.72 ± 0.26	5.66 ± 0.31	2.32 ± 0.23	41.29 ± 2.34	91.6 ± 2.2	0.75	1.14	1.89	4.97
Total		18	3.77 ± 0.26	5.70 ± 0.31	2.35 ± 0.23	41.71 ± 2.32	91.4 ± 1.9	0.74	1.12	1.86	5.07

* Average data lots B and D.
† Average data lots A and C.
Kruskal–Wallis test: no significative differences for IPP and ATP between the four lots, significative differences ($P < 0.01$) for P_i.
Jonckeere–Terpstra test for P_i data: significative difference ($P < 0.01$) for the alternative: $B \leq D \leq C \leq A$.

period (groups A and C) or in the middle December (group D) are tabulated in Table 4. Statistically significant differences existed among the three lots analyzed. For each muscle, with the highest values in heart, pectoral and wing muscle appearing in the post-arrival lot of birds (group C).

DISCUSSION

The hematological data reported by Kostelecka-Myrcha *et al.* (1972) on nestling starlings for the first 21 days, presented some values that are similar to our data on adult birds such as the hemoglobin content (17.1 g%) and hematocrit (49.9%), but the erythrocyte count was lower in nestling birds ($3.2 \times 10^6/mm^3$). Owing to this low count, the hematimetric indexes MCV and MCHb, were higher in young starlings.

In general, values in the hematocrit and in the hemoglobin content tend to increase in small sized birds as well as, in the more active flyers (Carey and Norton, 1976). Therefore, the hematological values found in starlings fit in well with the values found in other passeriformes, where the hematocrit ranged from 45% to 51%, and the hemoglobin concentration oscillated between 13 and 17.5 g% (Palomeque *et al.*, 1980a). Pigeons, powerful flyers with medium body size, also have a high hematocrit (46–52%) and hemoglobin (16–19 g%) concentrations (Palomeque and Planas, 1978; Ramis and Planas, 1978).

Also, the erythrocyte number in starlings, was closer to the small passeriformes 4–5.5 × $10^6/mm^3$ (Palomeque *et al.*, 1980a) than to the more frequent range in birds (2.5–3.5 × $10^6/mm^3$) according to Jones and Johansen (1972). However, the main corpuscular volume fitted in better with the values found in Galliformes (Balasch *et al.*, 1973) as well as the general values in birds (Jones and Johansen, 1972). The MCHb was also lower, like in Passeriformes (Palomeque *et al.*, 1980a), than the data in other groups, where the average values were between 40 and 55 pg Hb/erythrocyte (Jones and Johansen, 1972). The higher erythrocyte volumes in Passeriformes could explain the low MCHb values with the high hemoglobin concentrations.

Reticulocytes are present in starling blood, in a similar percentage to quails (Nirmalan and Robinson, 1972), pigeons and turkeys, but absent in chickens (Ramis and Planas, 1978).

The determination of the whole blood volume in starlings has also demonstrated another adaptation found in good flapping-flying species, where oxygen needs are very high. We have not found any data on small Passeriformes, but in pigeons the average values were 16.9–20.3 ml blood/100 g b.w. and the erythrocyte volume 8.3–9.8 ml erythrocytes/100 g b.w. (Palomeque and Planas, 1978), which is very close to starling data (Table 3). It is also known that an increase in blood volume is present in species with diving habits (Bond and Gilbert, 1958); but for most species of birds, blood content varied between 4 and 10 ml blood/100 g b.w. and 1.3 and 3 ml erythrocytes/100 g b.w. (Palomeque and Planas, 1978).

The hemoglobin oxygen affinity was higher than the values found in Passeriformes previously analyzed by us (Palomeque *et al.*, 1980a), where *Turdus merula*, with close body weights (86 g), presented a P_{50} of 52.4

Table 3. Blood volume and some oxygen transport properties in starlings (mean ± SE)

	Body weight (g)	Hc (%)	Blood volume (ml/100 g b.w.)	Erythrocyte volume (ml/100 g b.w.)	Oxygen affinity		Oxygen capacity (ml O_2 %)
					P_{50} (mmHg)	Bohr effect (P_{50}/pH)	
Total	66.8 ± 8.3 (27)	40.2 ± 6.9 (27)	16.95 ± 2.25 (27)	6.95 ± 1.41 (27)	38.1 ± 4.56 (10)	−0.472 ± 0.04 (5)	19.84 ± 3.35 (7)
Males (6)	62.4 ± 3.9	36.6 ± 6.6	17.32 ± 1.85	6.41 ± 1.74	—	—	—
Females (4)	57.8 ± 5.3	37.4 ± 5.8	17.03 ± 0.66	6.38 ± 1.11	—	—	—

Number of specimens analyzed in parentheses.

Torr, and the smaller Fringillidae, Sylvidae or Emberizidae, with body weights from 14 to 20 g, gave a P_{50} of 43–53.7 Torr. The number of specimens on that occasion was small, and in several species we had the data from only one specimen. This makes our speculations less useful, even though Lutz *et al.* (1974) suggested an inverse relationship between P_{50} and body weight.

The Bohr effect was quite uniform in Passeriformes (−0.403 to −0.532) and the values obtained in starlings fit into this range well, which is similar to other avian species (Palomeque *et al.*, 1980a; Lutz *et al.*, 1973; Meyer *et al.*, 1978; Baumann and Baumann, 1977).

In order to dig deeper in to the oxygen affinity of this species, we analyzed their main organic phosphates, ATP and IPP (inositol pentaphosphate, the main inositol polyphosphate in the starling, Riera *et al.*, 1983), plus their inorganic phosphate. We found (Table 2) similar levels in IPP as in other species, such as chickens, pigeons or ducks (Bartlett, 1978; Isaacks *et al.*, 1976; Borgese and Lampert, 1975), quails and swifts (Riera *et al.*, 1983). However, for the ATP we found high values in the starlings (5.5–5.9 μmol/ml RBC). The molar relationship IPP/Hb was 0.73–0.78 and was similar to data given by Isaacks and Harkness (1980) for adult Galliformes. However, the molar relationship IPP + ATP/Hb was higher (1.78–2.02) than the values published (1.0–1.4) by those authors for those species. High concentrations of ATP in the starling erythrocytes could explain this fact. ATP decreases oxygen affinity of avian hemoglobin (Borgese and Nagel, 1977; Isaacks *et al.*, 1977) but it increases the ATP/Hb ratio, which could cause the lowering of the hemoglobin affinity. However, the higher ATP levels in starlings are not reflected by specially high P_{50} values. The affinity of IPP to the bird hemoglobin is very high, but a connection between the variations of IPP concentrations and P_{50} values, is not conclusive (Pionetti and Bouverot, 1977; Petschow *et al.*, 1977; Black *et al.*, 1978). The similarity of IPP concentrations in several species (Riera *et al.*, 1983) offers some doubts on its modulator role. In the present study, no seasonal variations appeared and the molar ratio to Hb was constant. Lutz *et al.* (1980) suggested that the modulator effect of such organic phosphates could be achieved by a relative IPP/Hb variation rather than by absolute IPP level modifications. Seasonal differences in organic phosphate values were not enough to be considered as an adaptive trait (Table 2).

The direct measurements of the transport capacity in starling blood (Table 3), gave values near to theoretical capacity. With an average hemoglobin concentration of 15.29%, we obtained an oxygen transport capacity of 1.299 ml O_2/g Hb. This capacity is usually high in avian blood, with pigeons having the highest value (21.2% vol), and house sparrows or quails (Tucker, 1968; Baumann and Baumann, 1977) with a similar capacity to starlings.

In muscular organs, the myoglobin facilitates transport of oxygen from the capillaries to the mitochon-

Table 4. Myoglobin concentrations in several muscles in starlings. Body weight and the weight of the whole organs are also included (mean ± SE)

Group	Date of capture (*n* = specimens number)	Organs (muscles)					Mb total (mg)	Body weight (g)
		Heart	Pectoral	Wing	Legs			
					Gastrocnemius	Tight		
A	16 Nov. 1980 (*n* = 5)							
	Organ weight (g)	0.81 ± 0.12	21.89 ± 4.09	1.79 ± 0.26	2.26 ± 0.42	2.03 ± 0.30		65.0 ± 6.33
	Mb concentration (mg Mb/g wet)	2.07 ± 0.35	2.09 ± 0.31	2.05 ± 0.29	3.20 ± 0.57	2.36 ± 0.41		
	Mb content (mg Mb/organ)	1.66 ± 0.31	44.77 ± 6.23	3.69 ± 0.77	7.26 ± 2.03	4.70 ± 1.14	62.08	
C	2 Dec. 1981 (*n* = 6)							
	Organ weight (g)	0.81 ± 0.99	25.60 ± 1.93	2.60 ± 0.29	2.36 ± 0.34	2.28 ± 0.28		68.3 ± 3.70
	Mb concentration (mg Mb/g wet)	7.24 ± 2.16	9.54 ± 1.26	3.59 ± 0.67	1.12 ± 0.33	1.06 ± 0.29		
	Mb content (mg Mb/organ)	5.95 ± 1.54	244.89 ± 40.68	9.70 ± 1.72	2.65 ± 0.94	2.39 ± 0.70	264.46	
D	15 Dec. 1981 (*n* = 6)							
	Organ weight (g)	0.79 ± 0.07	24.40 ± 3.04	2.42 ± 0.31	2.46 ± 0.27	2.34 ± 0.20		65.6 ± 5.0
	Mb concentration (mg Mb/g wet)	3.29 ± 0.83	4.59 ± 2.25	1.56 ± 0.60	1.26 ± 0.49	0.68 ± 0.70		
	Mb content (mg Mb/organ)	2.63 ± 0.80	138.83 ± 87.88	3.64 ± 1.25	3.21 ± 1.56	1.61 ± 0.48	149.92	

* Both limbs included.

Kruskal–Wallis test: significant differences in the three lots (A, B and C) for each muscle.

Mann–Whitney U test: significant differences between the lots for each muscle, except: wing (A vs D) and gastrocnemius (C vs D).

dria, as was established by Wittenberg *et al.* (1975) in the pigeon breast. This fact has an adaptive character, because forced physical activity or obliged confinement provoked either an increase or a decrease, in the muscles affected (Lawrie, 1953; Catlett *et al.*, 1978; Pagés and Planas, 1983). The myoglobin content of the same muscle taken from different species was different, but clear-cut differences among the species according to their flying habits were difficult to establish (Pagés and Planas, 1983). So the values found in starlings are of the same type as in other species and only the aquatic birds, as a diving adaptation, presented concentrations that were 10–20 times higher (Deshpande, 1980; Weber *et al.*, 1974) while chickens with very low concentration in pectoral and wing muscles (Pagés and Planas, 1983) were clearly differentiated.

One of the aims of the present study was to look for any variations in oxygen transport properties of migratory starlings which could reflect the physical effort of the migration and possible variations during the winter stay. In respect to this, the effort of the migratory process seems evident in the myoglobin concentrations. Differences among the three lots analyzed (Table 4) were statistically significant in heart, wing and pectoral muscle, which could be due to the date of capture. A statistically significant reduction, about 50%, was observed from lot C (2 November 1981) to lot D (15 December 1981). The low total myoglobin content (67.08 mg) in lot A (16 November 1980) vs the 264.46 mg Mb in lot C could be explained by the differences in dates of arrival of both years. The stimulating effect of muscular activity during the migratory flight is partially lost during the stay in winter residences, where their flying activity is considerably reduced. The myoglobin reduction, especially in the pectoral muscle, in the post-migration state concurred with the results of experiments with pigeons in captivity, where this was evident after only 2 weeks of confinement (Catlett *et al.*, 1978; Pagés and Planas, 1983).

A statistical analysis carried out on the hematological data, reported in Table 1, looking for seasonal differences, did not show either a clear result or a trend that could be attributed to the migratory process.

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COMPARATIVE STUDY OF SOME INTESTINAL BRUSH BORDER MEMBRANE ENZYMES DURING PERINATAL DEVELOPMENT OF THE MOUSE*

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Abstract—1. Intestinal brush border membrane (bbm) fractions have been isolated from fetal and neonatal mice.

2. The existence of discordant developmental patterns of intestinal enzymatic activity derived from total homogenate and bbm fraction was confirmed.

3. It originates chiefly from two phenomena: (a) variations in the state of purity of brush border fractions, and (b) loss of brush border membrane enzyme activities in supernatant that increases with age.

4. The phenomenon of solubility for glucoamylase and alkaline phosphatase is already present two days before birth.

INTRODUCTION

The morphological and functional characteristics of adult small intestine are acquired following several events that occur during fetal and post-natal development. In rodents, as rat and mouse, the small intestine matures rapidly during the last 3 to 4 days of gestation so that its epithelium is ready to utilize milk at birth; subsequently, the intestinal mucosa will redifferentiate during the weaning period to be prepared for solid alimentation. This perinatal development has been reviewed by many authors (Moog, 1979, 1981; Henning and Kretchmer, 1973; Henning, 1981; Koldovsky, 1981a, b; Lebenthal and Lee, 1983).

Since the isolation of the brush border as a functional organelle of absorptive cells, numerous studies have confirmed the brush border as the site of terminal digestion and absorption leading to the concept of "digestive absorptive surface" (Crane, 1977). Patterns of enzymatic development, particularly for lactase, maltase and alkaline phosphatase, have been recently established on purified brush borders (Kedinger *et al.* 1981; Simon-Assman *et al.* 1982). As measured in total homogenates, brush border enzyme activities increase rapidly at the approach of birth (DeRitis *et al.*, 1975; Moog *et al.*, 1973; Calvert *et al.*, 1981). However, after birth, it seems that the developmental patterns differ when established from isolated brush borders or homogenates. Thus, several authors report that prenatal increase stops for alkaline phosphatase and maltase/glucoamylase activities (Moog, 1962; Moog *et al.*, 1973; Ménard and Malo, 1978) and slows down for lactase (Doell and Kretchmer, 1973; Moog *et al.*, 1973) when measured in homogenates. On the other hand, when the enzymatic activities are measured in isolated brush borders, alkaline phosphatase increases and disaccharidase activities drop dramatically (Kedinger *et al.*, 1981; Simon-Assman *et al.*, 1982) during the same period. During postnatal development, some brush border enzyme activities (especially lactase, maltase and alkaline phosphatase) are not completely associated with the brush border membrane and high amounts of brush border-like enzymes are recovered in supernatant fractions as compared to that recorded with adult small intestine (Galand and Forstner, 1974a, b; Seetharam *et al.*, 1977).

This work was undertaken in order (1) to elucidate the discrepancies reported for the enzymatic development when homogenates or brush border fractions are used and (2) to establish the developmental stages at which the increased solubilities for the different brush border enzymes appear.

MATERIALS AND METHODS

Animals

Swiss ICR strain mice were used. Mating was accomplished in our laboratory by leaving a male overnight with three females. Time of conception of embryos was fixed at 09.00 hr the next morning. The gestational period is 19 days. The second day after birth, the litters were reduced to eight animals and they were not fasted before sacrifice.

Preparation of tissue extracts

Fetal tissues were obtained from mice at 17 and 18 days of gestation. While the mother was maintained under ether anesthesia, fetuses were removed and the small intestine was excised. At 1 and 13 days after birth, the small intestine was removed immediately following decapitation. Pools of 5–8 entire small intestines were used at 17 and 18 days for the assays, whereas a pool of 2 intestines was used for each assay at 1 and 13 days after birth.

Isolation of brush border fractions

Isolation of brush border fraction was performed according to the classical technique of Schmitz *et al.* (1973). A 2% homogenate was made in 50 mM mannitol–2 mM Tris

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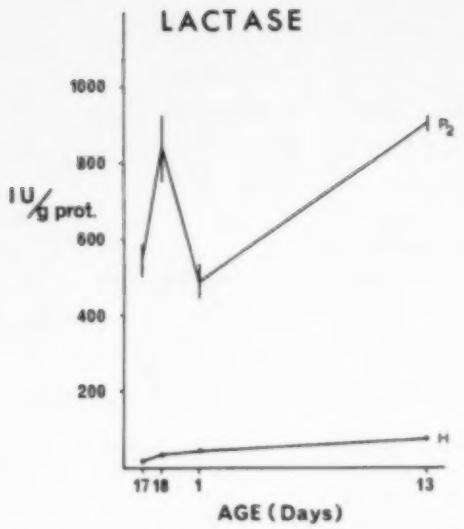

Fig. 1. Perinatal development of lactase activity established from homogenates (H) and brush border fractions (P_2). Each value represents the mean of six experiments (±SEM).

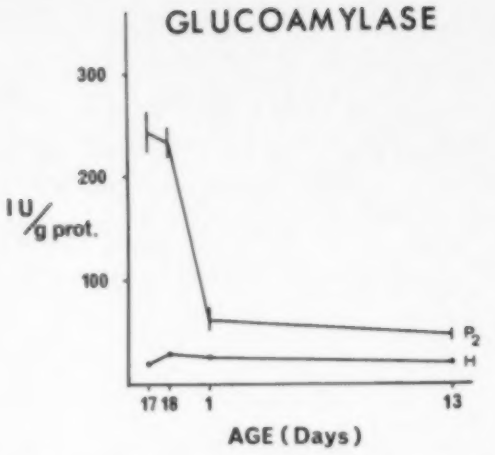

Fig. 2. Perinatal development of glucoamylase activity established from homogenates (H) and brush border fractions (P_2). Each value represents the mean of six experiments (±SEM).

(pH 7.1) at 4°C using an omnimixer (Sorvall) equipped with a microattachment, at full speed for two periods of 15 sec followed by one at 60 sec. $CaCl_2$ was added to a final concentration of 10 mM for 10 min. Centrifugation of the homogenate at 3000 *g* for 10 min gave a pellet (P_1) which was resuspended in 50 mM mannitol–2 mM Tris for further analysis, and a supernatant (S_1). S_1 was centrifugated at 27,000 *g* for 15 min and yielded a pellet (P_2) containing the brush border fragments and a supernatant (S_2). P_2 was resuspended in ice-cold redistilled water.

Solubility studies

Solubility of the brush border enzymatic activities was studied in supernatants obtained after brush border fragments isolation at 27,000 *g* (S_2) and in supernatants of homogenates of the same origin centrifuged at 100,000 *g* on Beckman L2-65 B with SW-50 rotor for 1 hr.

Assay method

Lactase activity was assayed according to a modification by Lloyd and Whelan (1969) of Dahlqvist's method (1964). Glucoamylase activity was measured using soluble starch as substrate according to Schlegel-Haueter *et al.* (1972) and alkaline phosphatase according to Eichholz (1967). Proteins were assayed according to Lowry *et al.* (1951).

RESULTS

Developmental patterns of intestinal enzymes

The developmental patterns for lactase, glucoamylase and alkaline phosphatase have been established using homogenates (H) and brush border fractions (P_2) obtained from mice small intestine at different developmental stages (17 days of gestation up to 13 days after birth). Lactase (L) activity rises significantly in H between 17 and 18 days ($P < 0.0005$), between 18 and 1 day ($P < 0.0005$) and between 1 and 13 days ($P < 0.005$). In P_2 fraction, L activity rises between 17 and 18 days of gestation ($P < 0.01$), decreases at 1 day ($P < 0.0005$) and rises again between 1 and 13 days ($P < 0.0005$) (Fig. 1). Glucoamylase (GA) exhibits an activity in H that rises between 17 and 18 days ($P < 0.005$) but then decreases between 1 and 13 days after birth ($P < 0.005$). Studied in P_2, this activity drops between 18 and 1 day ($P < 0.0005$) but remains stable during the first 13 days after birth (Fig. 2). Alkaline phosphatase (AlP) exhibits a third pattern of development. In H, its activity rises between 17 and 18 days ($P < 0.0005$), between 18 and 1 day ($P < 0.0005$) and between 1 and 13 days ($P < 0.0005$). In brush border membrane fraction (P_2), the activity decreases between 17 and 1 day ($P < 0.01$) but rises after birth ($P < 0.0005$) (Fig. 3).

Table 1 illustrates the protein contents of the brush border fractions isolated at different developmental stages. Also, the purification factors for the different brush border enzymes are reported. In P_2, the protein content is low at 17 days and increases at 18 days. One day after birth the percentage of total proteins found in P_2 is high but decreases slightly at 13 days after birth.

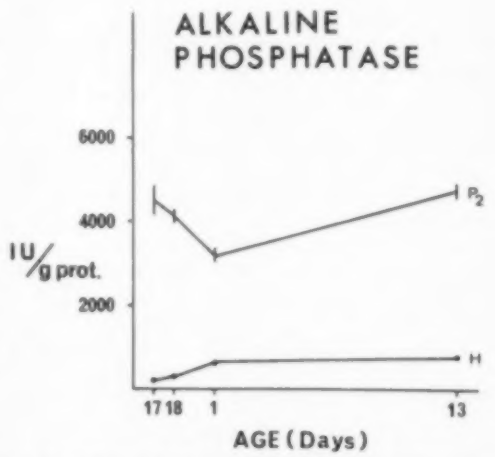

Fig. 3. Perinatal development of alkaline phosphatase activity established from homogenates (H) and brush border fractions (P_2). Each value represents the mean of six experiments (±SEM).

Table 1. Purification of brush border fraction

Age (days)*	Recovery of proteins (%) (P_2/H)	Purification factors (P_2/H) Lactase	Alkaline phosphatase	Glucoamylase
17 (8)†	1.01 ± 0.11‡	29.4 ± 2.8	21.1 ± 1.0	12.2 ± 0.8
18 (6)	2.05 ± 0.22	22.0 ± 1.8	13.1 ± 0.9	7.0 ± 0.4
1 (8)	6.80 ± 0.66	11.1 ± 0.7	5.1 ± 0.3	2.4 ± 0.4
13 (6)	4.12 ± 0.14	12.2 ± 0.6	6.1 ± 0.2	2.5 ± 0.2

* 17 and 18 days of gestation and 1 and 13 days after birth.
†Number of pools of entire small intestine assayed.
‡Mean ± SEM.

The purification is estimated by the raise of the specific activity of the enzymes in brush border membrane fraction. Thus, the three enzymatic activities show an increase of 12–29 times at 17 days in P_2 fraction in comparison with the specific activities present in H. These purification factors fall until 1 day ($P < 0.0005$) and remain stable between 1 and 13 days at 2.5 to 12.2, according to the different enzymes. Depending on the enzymatic activity, the total recoveries range between 84 and 104%.

Soluble fractions study

The analysis of S_2 fractions reveals that the percentage of L activity in S_2 is stable during perinatal development representing 15% of the total homogenate activity (Fig. 4a). On the other hand, the percentage of GA activity is quite significant at 17 and 18 days of gestation, representing 60% of the total activity. It increases up to 80% ($P < 0.005$) at 1 day and reaches 90% at 13 days ($P < 0.0025$). Finally 20% of the total AlP activity is found in S_2 fraction at the end of gestation. One day after birth, the soluble fraction represents 30% ($P < 0.0005$) and remains stable between 1 and 13 days.

In order to distinguish particulate from soluble enzymatic activities in S_2 fraction, equivalent homogenates were centrifuged at 100,000 *g* and the enzymatic activities determined in the supernatant fractions. Figure 4(b) shows that soluble GA activity before birth is close to 20% of total activity and increases approximately to 45% at 13 days after birth. For L, soluble activity remains low (3–7%) during the entire perinatal period. Soluble fraction of AlP during prenatal development is lower than 20% but increases gradually after birth up to 30% at 13 days.

DISCUSSION

Our results confirm the existence of discordant developmental patterns of some intestinal brush border enzymes such as lactase, glucoamylase and alkaline phosphatase. When specific activities are measured in total homogenate, they increase during

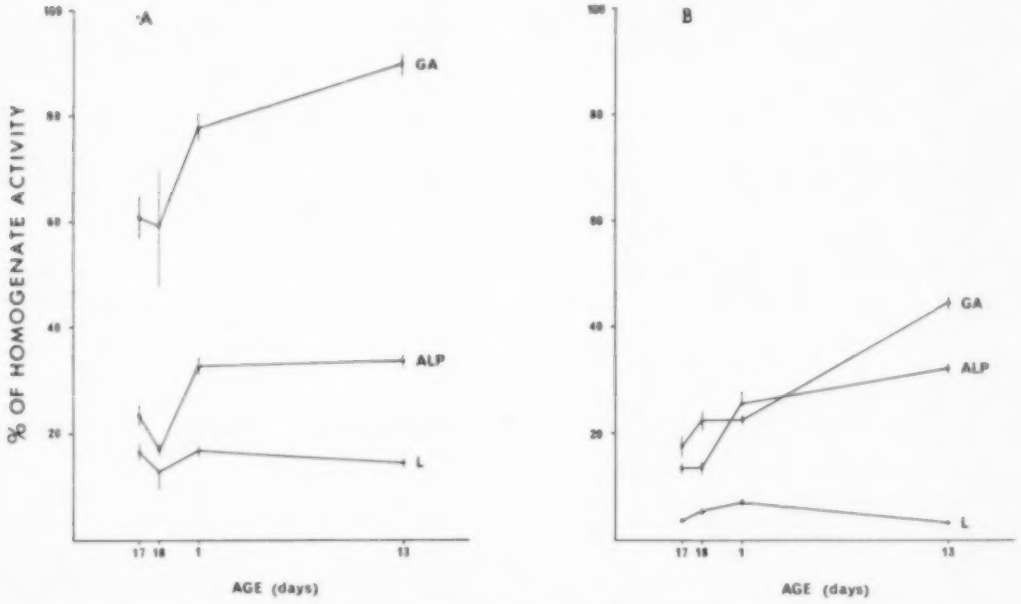

Fig. 4. Enzyme activities of glucoamylase (GA), alkaline phosphatase (AlP) and lactase (L) measured in 27,000 g (A) and 100,000 g (B) supernatants of homogenates of entire small intestines at 17 and 18 days of gestation and at 1 and 13 days after birth. Results are reported as % of total homogenate enzyme activity. Each value represents the mean of six experiments (± SEM).

the last days of gestation and slow down or stop after birth (Moog, 1962; Moog *et al.*, 1973; Doell and Kretchmer, 1973; DeRitis *et al.*, 1975; Ménard and Malo, 1978; Calvert *et al.*, 1981). When specific activities are measured in brush border fragments, they are high during gestation and fall at birth, especially for disaccharidases (Kedinger *et al.*, 1981; Simon-Asman *et al.*, 1982). In the subsequent days, patterns in brush border fractions and homogenates are alike: lactase and alkaline phosphatase increase and glucoamylase remains stable.

The study of the brush border fractions shows that the discrepancies between the developmental patterns can originate chiefly from two phenomena. The first one concerns the stage of purity of P_2 fraction isolated during different developmental stages. Even though the classical technique of Schmitz *et al.* (1973) can be applied successfully at any developmental stages and yield a brush border fraction, the state of purity differs considerably. Indeed, the purification factors for the different enzymes which are higher than that reported in adult (Schmitz *et al.*, 1973; Seetharam *et al.*, 1977; Berteloot and Hugon, 1982) fall at the approach of birth. After birth they remain stable and comparable to that reported for the same age in rats (Galand and Forstner, 1974b; Raul *et al.*, 1978). The variations of the purification factors result mainly from the variations of the proteins of the brush border fractions.

The second phenomenon concerns the percentages of the different enzymes remaining in the supernatant fractions and therefore they are not accounted for in the establishment of developmental patterns based on isolated brush border fractions. The present study clearly shows that the percentages of the different enzymatic activities remaining in the 27,000 *g* supernatant fraction vary for a given developmental stage but also change with the development. Studies with neonatal rat small intestine also reported an increased amount of soluble enzymes in supernatant fractions as compared to adult rat (Galand and Forstner, 1974; Seetharam *et al.*, 1977; Young *et al.*, 1981). This phenomenon already begins during the late fetal period. Even though the ultracentrifugation study indicates that the percentages of soluble enzymes are overestimated in the 27,000 *g* supernatant probably because of a smaller vesiculation of brush border membrane during homogenization, it confirms the same variations during the development especially for GA and AlP. The observed stability for L activity agrees with previous studies on rat small intestine (Young *et al.*, 1981).

In conclusion, the discrepancy observed between the developmental patterns established either on homogenates or brush border membrane fractions results mainly from the state of purity of the brush border membrane fraction which varies considerably depending on the studied developmental stages and on the solubility of each enzyme, phenomenon already present two days before birth. Isolation of intestinal brush borders may present some advantages over homogenates in the study of enzymatic development under hormonal treatments or in organ culture. However one must be cautious in the interpretation of developmental patterns in applying the classical technique of Schmitz *et al.* (1973) to fetal and neonatal intestine without modification.

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VENTILATORY CHANGES IN THE GOLDEN HAMSTER, *MESOCRICETUS AURATUS*, COMPARED WITH THE LABORATORY RAT, *RATTUS NORVEGICUS*, DURING HYPERCAPNIA AND/OR HYPOXIA

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Abstract—1. Tidal volume, respiratory frequency, and minute volume were measured with total body plethysmography.

2. Both hypercapnic (0–7% CO_2) and hypoxic (13–21% O_2) gas mixtures caused "dose" related hyperventilation in the hamsters. However, there was no synergism between combined stimuli.

3. Rats exhibited greater hyperventilation than the hamsters during hypercapnic + hypoxic and hypercapnic exposures. Hamsters responded more than the rats to hypoxia alone.

4. Greater blood buffering capacity of hamsters is a possible explanation for the species differences in ventilatory sensitivity.

INTRODUCTION

Small mammal burrows provide shelter from predators and have stable air temperatures and high relative humidities. While most burrow conditions are relatively constant, gas concentrations often vary considerably more than in outside air (Baudinette, 1974).

Compared to atmospheric air, carbon dioxide levels of burrows are typically high (hypercapnic) and oxygen levels are low. This results from poor circulation of air and respiration of the animal, soil microorganisms, soil flora and roots of surface plants (Chapman and Bennett, 1975). For example, in mole rat (*Spalax ehrenbergi*) burrows, oxygen was found to range from 17.4 to 20.9% and carbon dioxide ranged between 0.6 and 2.8% (Arieli, 1979). Gas composition of pocket gopher (*Thomomys bottae*) burrows ranged from 6.0 to 21% oxygen and 0.6 to 3.8% carbon dioxide (Darden, 1972).

Generally, fossorial mammals tolerate elevated CO_2 levels better than nonburrowing forms (Chapman and Bennett, 1975; Arieli and Ar, 1979). Heart rate and body temperature declines associated with high levels of carbon dioxide are much less in burrowing animals (Soholt *et al.*, 1973). Additionally, oxygen consumption is relatively unaffected by exposure to hypoxic and/or hypercapnic environments (Ar *et al.*, 1977). Fossorial mammal blood typically has a higher hematocrit, hemoglobin concentration, oxygen affinity and blood buffering capacity than found in nonburrowing species (Hall, 1966; Lechner, 1976). All these characteristics are adaptive in a burrow which has a hypercapnic–hypoxic environment.

Ventilatory changes in response to hypercapnia are apparently low for fossorial mammals in comparison with those exhibited by nonburrowing species. Darden (1972) exposed pocket gophers to carbon dioxide concentrations ranging from 2 to 8% and showed that the CO_2 response was less than that seen in man, echidnas, seals and manatees. The strictly fossorial mole rat (*Spalax ehrenbergi*) was much less sensitive to high carbon dioxide or low oxygen when compared to the Wistar laboratory rat (Arieli and Ar, 1979).

With one exception (Arieli and Ar, 1979), studies on ventilatory control in fossorial mammals have used changes in carbon dioxide only. The objective of this study was to compare the ventilatory responses of the golden hamster, *Mesocricetus auratus* and *Rattus norvegicus* (Sprague–Dawley strain) during exposure to hypercapnic and/or hypoxic gas mixtures. Hamsters, unlike the rats, hibernate during cold periods of the year in a completely or partially sealed burrow in which the hamster undergoes frequent arousal (Pohl, 1965). The combined effect of arousals and the sealed burrow probably results in significant alterations of the CO_2 and O_2 concentrations although no direct measures for hamsters have been published. The hypothesis to be tested then was that golden hamsters are better adapted to hypercapnic and/or hypoxic conditions, and therefore show less of a ventilatory change under these conditions than the white rat.

MATERIALS AND METHODS

Animals

Ten adult male golden hamsters weighing 115.4 g ± 11.0 (SD) were used in this study. They were purchased from Haley Farm (Quality Research Animals) in Hurt, VA. Five adult male Sprague–Dawley rats, weighing 435.0 g ± 40.6 (SD), were used for comparison. The rats were obtained from Dominion Research Labs in Dublin, VA. All animals were

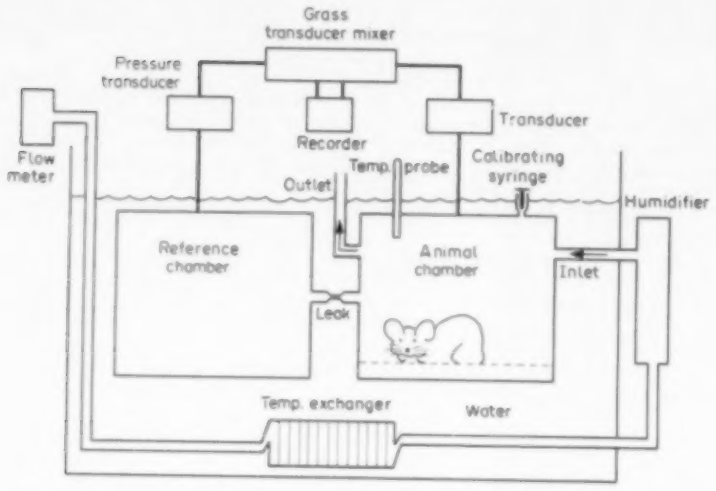

Fig. 1. Plethysmograph apparatus used to measure ventilation in hamsters and rats. See text for explanation.

housed singly in polystyrene cages (29L × 19W × 13 cm H), and were fed Wayne lab chow and water *ad libitum*. The hamsters were maintained in a room with 15L–9D photoperiod, while the photoperiod for the rats was 14L–10D. The hour difference was not intentional, but was the result of lodging constraints.

Gas mixtures

The following gas mixtures were used in this work: hypercapnic–hypoxic mixtures of 3% CO_2–17% O_2, 5% CO_2–15% O_2, and 7% CO_2–13% O_2; hypercapnic mixtures of 3% CO_2 and 7% CO_2; and hypoxic mixtures of 17% and 13% O_2. Nitrogen served as the balance gas in all cases. These particular gas concentrations were chosen because they approximate those measured in burrows (Arieli, 1979).

Gas mixtures were prepared from commercial gas cylinders to which flow meters were attached. Tygon tubing led from each flow meter into a common tube where the gases were mixed. This tubing was connected to the experimental chamber. The gas concentrations of a particular mixture were checked frequently during each experiment with a Scholander Gas Analyzer (Scholander, 1942).

Experimental apparatus

Total body plethysmography was used to measure tidal volume, respiratory rate, and minute volume in hamsters and rats (Bartlett and Tenney, 1970; Malan, 1973). The plethysmograph chambers consisted of two Plexiglass 1650 cm^3 cylinders, 27.3 cm long and 8.9 cm in dia.

One chamber contained the animal, while the other served as a thermobarometer or reference chamber. Each was sealed with rubber stoppers. The experimental chamber was equipped with gas inlet and outlet tubes, a temperature probe and a 1 cm^3 calibrating syringe. This chamber was connected to the reference chamber by a short piece of tygon tubing to which a hypodermic needle was attached. This served as a slow leak, which prevented the pressure record from drifting due to possible small temperature changes in the animal chamber. Each chamber was connected to a Statham Model P23BC pressure transducer which was fed into a GRASS Model TM-1 transducer mixer. The differential pressure changes were then recorded on a GRASS Model 5D polygraph (Fig. 1).

Experimental procedures

It was observed early in the study that when the gas mixture was changed a large hyperventilation occurred, but then over a period of time, the ventilation returned to an intermediate level at which it tended to stabilize. Preliminary experiments were run for each gas mixture to determine the length of time required for this plateau to take place after the mixture was changed. These were done by exposing a hamster to a particular gas mixture for 3 hr during which time measurements were taken every 10–15 min (Table 1).

For all the measurements in this study, the animal was unrestrained in the experimental chamber. This chamber and the reference chamber were maintained in a water bath at thermalneutrality (28–30°C). The zone of thermalneutrality for the hamsters was predetermined by measuring oxygen consumption over a wide range of temperatures. When ventilation measurements were made, the inlet and outlet tubes were clamped off, and the pressure changes due to the hamster's breathing were recorded for approximately 15–20

Table 1. Composition of gas mixtures used throughout this study and the time duration required for the ventilatory response of the hamsters and rats to stabilize after being exposed to that mixture

Gas mixture	Partial pressure P_{CO_2}	P_{O_2} (Torr)	Time (min)	Hamster sample size (n)	Rat sample size (n)
G1 (3% CO_2, 17% O_2)	20.2	114.5	30	5	0
G2 (5% CO_2, 15% O_2)	33.7	102.0	60	5	0
G3 (7% CO_2, 13% O_2)	47.1	87.5	120	5	5
G4 (3% CO_2, 21% O_2)	20.2	141.4	60	8	0
G5 (7% CO_2, 21% O_2)	47.1	141.4	120	8	5
G6 (0% CO_2, 17% O_2)	0	114.5	30	7	0
G7 (0% CO_2, 17% O_2)	0	87.5	90	5	5

sec. Also at this time, for calibration, 0.2 cm^3 of air was injected into the chamber and the resulting pressure change recorded.

Hamsters were randomly exposed to the seven hypercapnic and/or hypoxic gas mixtures but only three mixtures during any one day. Typical experimental procedure was as follows: if a hamster was to be exposed to gas mixtures 1, 2 and 3, it was first placed in the experimental chamber for 1 hr. Air was flushed through the chamber and ventilation was recorded after 45 and 60 min. Next, the hamster was exposed to gas mixture 1, and ventilation was recorded after 30 min of continuous exposure. Turnover time was taken into account during this time duration. It was calculated and experimentally confirmed to be less than 2 min. Air was then flushed through the chamber and ventilation was recorded after 15 and 30 min of exposure. After this, the hamster was exposed to gas mixture 2 for 60 min and ventilation was recorded at this time. The experiment proceeded in this manner with the hamster being exposed to air before exposure to a specific hypercapnic and/or hypoxic gas mixture. Time duration of an experimental run was usually no more than 5 hr. Flow rate for all gas mixtures was 6500 cm^3/minute.

Experimental protocol for the rat study was the same as that of the hamsters, except the rats were exposed only to the extreme CO_2 and low O_2 gas mixtures. A thermalneutral zone between 28 and 30°C was also used for the rats (Baker *et al.*, 1980).

Tidal volume was calculated from the equation by Drorbaugh and Fenn (1955) using measurements from the strip chart records. Respiratory frequency was calculated by counting the number of pressure deflections or "breaths" over a 15–20 sec span on the record. Minute volume was determined by multiplying tidal volume by respiratory frequency (per min).

The raw data for a particular hypercapnic and/or hypoxic gas mixture were compared with the values obtained for air (the control gas mixture) and the differences recorded. The changes in the ventilatory parameters for the hamsters were analyzed statistically using two way ANOVA with missing cells and Fischer's Least Significant Difference (LSD) test. The rat data were analyzed using two way ANOVA and Duncan's Multiple Range test. Statistical comparison of the rat and hamster data was done using ANOVA (Ott, 1977). In interpretation of the results, changes are significantly different if $P < 0.05$. The statistical analyses comparing the responses to gas mixtures are shown in Tables 2 and 3.

RESULTS

Hamster

Hamsters responded to the hypercapnic–hypoxic gas mixtures by increasing their minute volume ($\dot{V}_E$) as conditions became more extreme (Tables 2 and 4, Fig. 2). Percent tidal volume increases were approximately twice as great as those of respiratory frequency (Tables 4 and 5).

Hypercapnic gas mixture, G5 (7% CO_2; 21% O_2) produced a greater change in $\dot{V}_E$ than that of G3 (3% CO_2, 21% O_2) but neither produced a response significantly larger than that caused by comparable hypercapnic mixtures that were also hypoxic (Tables 2 and 4, Fig. 2).

For hypoxia alone, minute volume change increased as the gas mixture became more hypoxic. The response to hypoxia (G6 and G7) indicated that the hamsters were as sensitive to this stressor alone, as when it was in combination with elevated CO_2 (G1 and G3). The increase in minute volume was due to a rise in respiratory frequency alone; tidal volume changes

Table 2. Statistical analysis of hamster and rat experimental results

Gas mixture comparisons	Change in tidal volume	Statistics* (*P* value)	Change in respiratory frequency	Statistics* (*P* value)	Change in minute volume	Statistics* (*P* value)
			Hamster			
G1, G2, G3	G3 > G1	<0.0001, <0.0001	G3 > G1	<0.0001, <0.03	G3 > G2 > G1	<0.0001, <0.0001 (G3 > G1); <0.0001, <0.002 (G3 > G2); <0.0001, <0.003, (G2 > G1)
	G1 ≠ G2†	–	G1 ≠ G2	–		
	G2 ≠ G3	–	G2 ≠ G3	–		
G4, G5	G5 > G4	<0.0001, <0.0072	G4 ≠ G5	–	G5 > G4	<0.0001, <0.0003
G6, G7	G6 ≠ G7	–	G7 > G6	<0.0001, <0.0001	G7 > G6	<0.0001, <0.0001
G1, G4, G6	G6 < G1 and G4	<0.0001, <0.0001	G6 > G1 and G4	<0.0001, <0.0001	G1 ≠ G4 ≠ G6	–
G3, G5, G7	G7 < G3 and G5	<0.0001, <0.0001	G7 > G3 and G5	<0.0001, <0.0001	G7 > G3 and G5	<0.0001, <0.005 (G7 > G5); <0.0001, <0.0001 (G7 > G3)
	G3 ≠ G5	–	G3 ≠ G5	–	G3 ≠ G5	–
			Rat			
G3, G5, G7	G7 < G3 and G5	<0.01, <0.05	G3 ≠ G5 ≠ G7	–	G7 < G3 and G5	<0.001, <0.05

*2-way ANOVA with missing cells and no interaction (note that the assumption of no interaction was fairly accurate because a 2-way ANOVA on two of the hamsters which were exposed to all of the gases—no missing cells—gave no significant interaction), Fisher's Least Significant Difference.

†No significant statistical difference.

‡2-way ANOVA, Duncan's Multiple Range Test.

Table 3. Statistical analysis comparing experimental results of the hamster with the rat

Gas mixture	Change in tidal volume	ANOVA	Change in respiratory frequency	ANOVA	Change in minute volume	ANOVA
3 (7% CO_2, 13% O_2)	Rats higher	$P < 0.0001$	Rats higher	$P < 0.0001$	Rats higher	$P < 0.0001$
5 (7% CO_2, 21% O_2)	Rats higher	$P < 0.0025$	Rats higher	$P < 0.0001$	Rats higher	$P < 0.0001$
7 (0% CO_2, 13% O_2)	Rats higher	$P < 0.01$	Hamsters higher	$P < 0.03$	Hamsters higher	$P < 0.02$

did not contribute to the increase seen (Tables 2 and 4, Fig. 2).

Rat

Minute volume changes for the hypercapnic–hypoxic gas mixture (G3) and the hypercapnic gas mixture (G5) were similar (i.e. hypoxia did not potentiate the hypercapnic response). Hypoxia (G7) alone caused less effect on ventilation than elevated CO_2 (G5) alone (Tables 2 and 4). Hypercapnia caused greater changes in tidal volume than in respiratory frequency, whereas hypoxia stimulated both parameters almost equally (Tables 4 and 5).

Comparative hamster vs rat

Rats were more responsive to hypercapnic–hypoxic gas mixture 3 and the hypercapnic gas mixture 5 than the hamsters. Changes in V_T, R_f, and $\dot{V}_E$ shown by the rats were all greater than that of the hamsters. Hypoxia alone (G7) produced larger R_f and $\dot{V}_E$ changes in the hamsters. Tidal volume change, however, was less for the hamsters than the rats (Tables 3 and 4, Fig. 3).

DISCUSSION

Control (air) values

The respiratory values obtained on the rats are in close agreement with predicted values in the literature, which are based on body weight formulas (Table 5). However, those for the hamster differ considerably from the predicted values for mammals in general. Hamster tidal volume was nearly twice the predicted values, while respiratory frequency and minute volume were smaller (Table 5). Both the rats and hamsters were

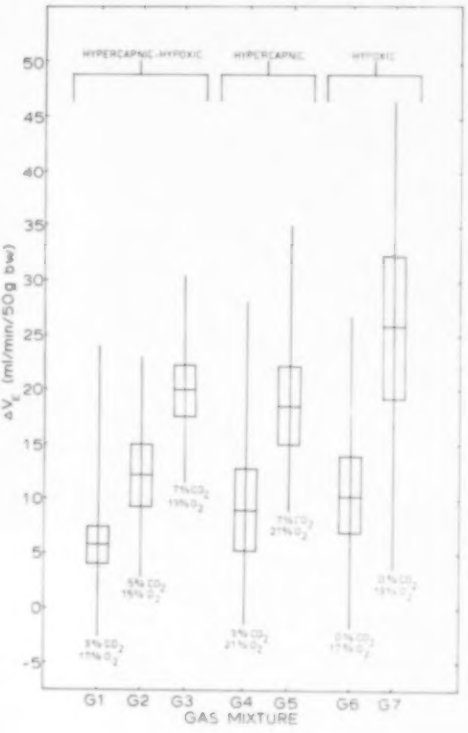

Fig. 2. Hamster — mean change in minute volume with respect to air for designated gas mixtures. The boxes represent ±2 SE, and the vertical lines the range.

Table 4. The average change ±2 SE in the ventilatory variables with respect to air for a designated gas mixture and the range

Gas mixture	Mean change in tidal volume ($\Delta V_T \pm 2$ SE)*	Range	Mean change in respiratory frequency ($\Delta R_f \pm 2$ SE)	Range	Mean change in minute volume† ($\Delta \dot{V}_E \pm$ SE)	Range
			Hamster			
G1 (3% CO_2, 17% O_2)	0.30 ± 0.13	−0.45–+0.87	2.75 ± 1.89	−12–+12	5.65 ± 1.67	−2.60–+23.90
G2 (5% CO_2, 15% O_2)	0.63 ± 0.19	0–+1.25	5.00 ± 2.33	−6–+15	12.09 ± 2.87	2.7–+22.80
G3 (7% CO_2, 13% O_2)	0.95 ± 0.15	0.51–+1.69	8.61 ± 3.04	−5–+20	19.89 ± 2.35	11.4–+30.40
G4 (3% CO_2, 21% O_2)	0.81 ± 0.33	−0.59–+2.68	−0.76 ± 3.66	−15–+15	8.96 ± 3.74	−1.45–+28.10
G5 (7% CO_2, 21% O_2)	1.26 ± 0.42	−0.17–+3.44	3.20 ± 3.36	−15–+15	18.58 ± 3.55	8.90–+35.00
G6 (0% CO_2, 17% O_2)	−0.37 ± 0.27	−1.60–+0.51	22.08 ± 6.04	6–+47.5	10.32 ± 3.44	−1.60–+26.70
G7 (0% CO_2, 13% O_2)	−0.15 ± 0.31	−1.30–+0.90	41.41 ± 8.17	20–+82.5	25.74 ± 6.50	3.60–+46.50
			Rat			
G3 (7% CO_2, 13% O_2)	2.10 ± 0.55	−0.14–+4.15	39.30 ± 12.19	−18.8–+75	42.00 ± 6.79	25.60–+7.13
G5 (7% CO_2, 21% O_2)	2.54 ± 0.71	0.05–+5.02	28.57 ± 11.80	−10–+60	37.69 ± 5.32	18.90–+56.80
G7 (0% CO_2, 13% O_2)	1.09 ± 0.88	−1.73–+5.60	23.20 ± 14.53	−20–+90	15.94 ± 4.43	−3.60–+22.93

*SE = standard error.
†Mean change in minute volume is based on 50 g body weight.

Table 5. Control air ventilatory values derived from 146 hamster trials and 46 rat trials. Other data from the literature given for comparison

Tidal volume (ml)	Respiratory frequency (breaths/min)	Minute volume (ml/min)	Source
		Hamster	
1.61 ± 0.46 (2 SE)	26.7 ± 6.3 (2 SE)	42.02 ± 2.09 (2 SE)	Measured in this study
0.85	90.00	76.50	Predicted (Guyton, 1947)
0.81 ± 0.37 (SE)	93.80 ± 37.52 (SE)	67.40 ± 30.33 (SE)	Predicted (Stahl, 1967)
(0.44–1.18)*	(56.28–131.32)	(37.07–97.73)	
1.03	32	33	(Chapin, 1954)
0.83	74	54	(Spector, 1956)
(0.42–1.2)	(33–127)	(25–83)	
0.66 ± 0.2 (SD)	76 ± 5.0 (SD)	50 ± 14 (SD)	(Mauderly *et al.*, 1979)
1.11 ± 0.33 (SD)	66.9 ± 17.9 (SD)	70.9 ± 14.5 (SD)	(Javaheri *et al.*, 1980)
0.68 ± 0.09 (SD)	44.5 ± 14.0 (SD)	29.3 ± 10.4 (SD)	(Strope *et al.*, 1980)
		Rat	
3.05 ± 0.79 (2 SE)	69.32 ± 18.29 (2 SE)	206.11 ± 16.82 (2 SE)	Measured in this study
3.22	64.6	208	Predicted (Guyton, 1947)
3.24 ± 1.46 (SE)	66.43 ± 26.57 (SE)	194.70 ± 87.62 (SE)	Predicted (Stahl, 1967)
(1.78–4.70)	(39.86–93.00)	(107.09–282.32)	
1.55	97	61	(Crosfill and Widdicombe, 1961)†
(1.03–2.13)	(84–126)	(46–85)	
1.84 ± 0.21 (SD)	113.7 ± 10.9 (SD)	224.9 ± 27.9 (SD)	(Leong *et al.*, 1963)†
2.63 ± 0.47 (SD)	84.9 ± 1.66 (SD)	223.3 ± 44.19 (SD)	(Bartlett and Tenney, 1970)
1.17 ± 0.35 (SD)	78.4 ± 11.2 (SD)	92.9 ± 30.7 (SD)	(Arieli and Ar, 1979)†
0.86	85.5	73	(Baker *et al.*, 1980)
(0.60–1.25)	(66–114)	(50–101)	

*Range.
†All the rats in the studies cited weighed substantially less (300–400 g) than the rats in this experiment.

subjected to the same experimental protocol, so this is not apparently due to apparatus. Perhaps part of the difference observed in the ventilatory variables of the hamsters resulted from specific physiological adaptations. Predicted values for mammals do not account for special adaptations which may have evolved within a species (Stahl, 1967). Indeed, most of the minute volume measurements obtained by others on hamsters are lower than would be predicted (Table 5).

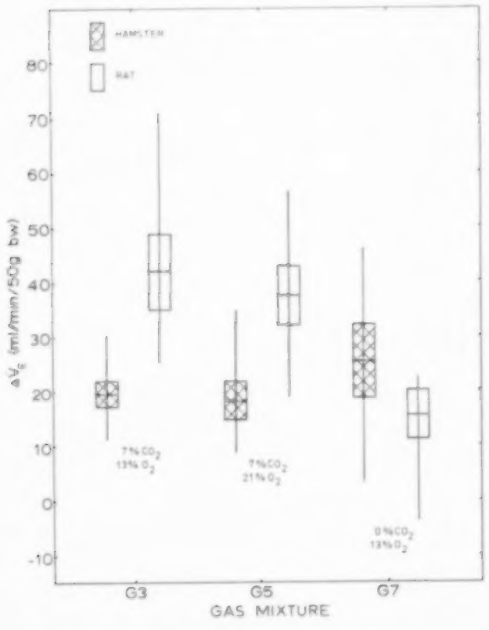

Fig. 3. Comparison of the mean change in minute volume during gas mixture exposure with respect to air for the hamster with that of the rat. The boxes represent ± 2 SE, the vertical lines the range.

There is considerable variation in hamster respiratory frequency and tidal volume values reported in the literature (Table 5). Those obtained in this study are at the two extremes of the range: the tidal volume was higher and the respiratory frequency lower than reported by others. There does not appear to be an obvious explanation for this difference.

The source of variation in the literature values for $\dot{V}_E$ is probably the experimental temperatures. With the exception of Chapin (1954), most of the measurements have been made at temperatures below the thermalneutral zone (or the temperature was not given). Homeotherms increase oxygen consumption, and thus $\dot{V}_E$ at temperatures below the thermalneutral zone.

Ventilatory variables measured for the rat also differ from some literature values. For example, tidal volume measured in this study was twice those previously reported. As tidal volume is proportional to weight to the power of 1.04 (Stahl, 1967), then as weight increases so would this ventilatory variable. Bartlett and Tenney (1970) had ventilatory parameter values similar to those of this study (Table 5) which is expected since their rats were the only ones of comparable weight to those used here.

Hamster ventilatory responses to hypercapnia and/or hypoxia

Chapin (1954) and Javaheri *et al.* (1980) have studied the ventilatory response of golden hamsters to varying

levels of carbon dioxide using the plethysmographic technique. Chapin reported his data as ventilation ratio (minute volume during CO_2 exposure: minute volume of the same animal breathing air) vs percentage CO_2. When his hamsters were exposed to 3% CO_2 the ratio was approximately 1.5 and for 7% CO_2 it was about 2.5. Ventilation ratios for this study were similar at 3% CO_2 (1.56) but slightly lower in 7% CO_2 (1.97). Chapin assumed that 20–30 min exposure time was sufficient to reach a steady state. Our research indicated that 120 min was required. Therefore, the higher ventilation ratio reported by Chapin at 7% CO_2 may have been due to ventilatory overshoot before steady state was reached. However, Javaheri *et al.* (1980) used only a 15-min exposure to hypercapnic gas mixtures ranging from 5 to 10% CO_2. The tidal volume and respiratory rate changes recorded during exposure to 7% CO_2 were similar to those measured in this study, and both showed an approximate doubling in minute volume. It would seem that different hamster strains require different periods of time to adjust to a changed gas concentration, a problem that has been apparently overlooked in most studies.

The increase in minute volume under hypercapnia was achieved more by an elevation in tidal volume than in respiratory frequency in both Javaheri's and this study. In contrast, respiratory rate change was greater than that of tidal volume in the Chapin (1954) study. At present there seems to be no good explanation for this difference.

Hypoxia, as an additional respiratory stressor, often potentiates the ventilatory response to CO_2 in mammals such as the cat, dog and man (Comroe, 1965; Arieli and Ar, 1979). In the cat, there is a positive interaction between hypoxia and hypercapnia on the discharge rate of the carotid body (Fitzgerald and Parks, 1971). However, ventilatory responses of the hamsters to the hypercapnic–hypoxic gas mixtures (G1 and G3) were not significantly different from that of the respective hypercapnic gas mixture of identical CO_2 concentration (Table 2, Fig. 2). Similar findings were also reported for the mole rat, a strictly fossorial species (Arieli and Ar, 1979). Thus, there appears to be a lack of synergism with these two respiratory stressors in the fossorial species studied so far.

Hypoxia alone produced greater respiratory frequency and minute volume changes than those produced by similar hypoxic–hypercapnic gas mixtures. It seems then that the carotid body in the hamster is sensitive to hypoxia, but elevated CO_2 may act as a suppressor, instead of an enhancer of the respiratory response. The higher buffering capacity of the hamster's blood (to be discussed below) may contribute to this effect.

Rat ventilatory responses to hypercapnia and/or hypoxia

Exposure of the rats to hypercapnia produced a greater percent increase in tidal volume than in respiratory frequency (Table 4). Evidently, as in the hamsters there is some variation in how rats respond to hypercapnia. Bartlett and Tenney (1970) and Arieli and Ar (1979), using total body plethysmography, found an approximately equal increase in these two variables. The minute volume change Bartlett and Tenney (1970) measured was greater than the change recorded in this study. The difference can be attributed to the shorter exposure time which was probably insufficient to achieve a steady state.

The response of Bartlett and Tenney's (1970) rats to hypoxia was also larger than measured in this study. A lower oxygen concentration (10% compared to 13%) and a shorter exposure time probably contributed to the observed difference.

Ventilatory responses of the rats in this study to hypoxia and hypercapnia together were not significantly different from the response to hypercapnia alone (Table 2). This differs from the findings of Arieli and Ar (1979) who observed that the ventilation response of the rats to hypoxia was potentiated by hypercapnia. They exposed their animals to a wider range of hypoxic conditions which was more extreme than the 13% O_2 used in our study. Thirteen percent oxygen may not have been a strong enough stimulus to elicit a large ventilatory response. For example, man does not show a strong ventilatory response to hypoxia until the oxygen concentration is less than 9% (Comroe, 1965). Perhaps if the hypoxic conditions had been more severe a potentiation in the ventilatory response to hypercapnia and hypoxia would have been observed.

Comparison of hamster ventilatory responses to hypercapnia and/or hypoxia with that of the rat

The ventilatory responses of the hamster to the hypercapnic–hypoxic gas mixture, G3, and the hypercapnic gas mixture, G5, indicate less responsiveness to carbon dioxide when compared to the rat (Table 2, Fig. 3). Minute volume of the hamsters increased by only 210% when exposed to G3 and 198% when exposed to G5, compared with the minute volume increase by the rats of 270% and 264%, respectively. This is probably due in part to differences in their blood buffering capacities. Javaheri *et al.* (1980) found the $[HCO_3^-]$ of hamsters to be 30.8 + 2.5 mmol/l, while the rat has a $[HCO_3^-]$ of only 19.8 mmol/l (Chapman and Bennett, 1975). This greater buffering capacity of the hamsters would reduce the hydrogen ion change produced by breathing hypercapnic gas mixtures, so the stimulus for breathing would be less in this species. Darden (1972) also believes that the increased buffering capacity of fossorial rodents is a possible explanation for the observed carbon dioxide "insensitivity". Additionally, he suggests that there may be a decreased "sensitivity" of respiratory control receptors of fossorial rodents to change in blood pH.

Hamsters increased their minute volume by 233% after exposure to 13% O_2 compared with a 167% increase in the rat. This difference in ventilatory response to hypoxia may also be due to the greater buffering capacity of the hamsters. As a mammal inspires air with a low oxygen tension, minute volume increases and carbon dioxide decreases in alveolar air and arterial blood. Thus, the carbon dioxide and hydrogen ion stimuli for breathing are lowered and minute volume may decrease even though a hypoxic condition exists (West, 1979). The hamster's blood, due to its greater buffering capacity would not become as alkalotic as the rat's. Therefore, minute volume increase of the hamster in response to hypoxia would be greater than the rat's, which was observed in this study.

Another factor which may give further explanation for the differing responsiveness to carbon dioxide that exists between two species is that the hamster is a hibernator. During its hibernation bouts, the burrow entrance is partially or completely sealed, which probably results in extreme hypercapnia or hypoxia (Pohl, 1965). Lyman (1951) found that hibernating hamsters exhibit less of an increase in respiratory frequency when breathing CO_2 than nonhibernating hamsters, although the results were highly variable. And Briorck *et al.* (1956) observed that hibernating hedgehogs did not increase their respiratory rate as much as nonhibernating ones when both were exposed to 6 and 9.5% CO_2. A study similar to Biorck *et al.* (1956) on hamsters would be of interest to determine if there is a decreased responsiveness to hypercapnia and/or hypoxia during hibernation in hamsters.

In conclusion, the hamsters exhibited less of a ventilatory response to carbon dioxide than the rats. However, hamsters were more responsive to hypoxia than the rats. The differing ventilatory responses may be largely explained by the greater blood buffering capacity of the hamster.

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INTERRELATIONSHIPS OF CONSTITUENTS AND PARTITION OF SALTS IN MILK SAMPLES FROM EIGHT SPECIES

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Abstract—1. Skim-milk samples from rat, rabbit, pig, sheep, goat, horse and man were analysed for lactose, casein and the total and ultrafilterable concentrations of the main salts. Results are compared with data for the cow.

2. The ultrafiltrate concentrations of Ca and Mg were positively correlated with that of citrate and the colloidal concentrations of Ca, Mg and citrate were positively correlated with that of P_i, suggesting that common, general, principles determine the partition of salts in milks.

3. Casein concentration in the skim-milks was inversely related to that of lactose in accordance with a recent theoretical treatment of the principal mechanism of milk secretion.

4. It is postulated that much of the interspecific diversity in milk composition can be explained by adaptations in a single secretory mechanism.

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INTRODUCTION

Concentrations of fat, protein and carbohydrate in milks of over a hundred species have been reported (Jenness and Sloan, 1970), but salt composition has been investigated less fully (Jenness, 1974; Katoaka *et al.*, 1972). For example, detailed information on the partition of salts into colloidal and ultrafilterable fractions has been obtained only for the cow, *Bos taurus* (White and Davies, 1958) and water buffalo, *Bubalus bubalis* (Abd El-Salam and El-Shibiny, 1966; Albonico *et al.*, 1969), with incomplete studies reported for some other domesticated species (O'Connor and Fox, 1977; Ormrod *et al.*, 1982; Puri and Parkash, 1965) and man, *Homo sapiens* (Kondo *et al.*, 1964).

Studies on cow milk by equilibrium dialysis and ultrafiltration have shown that approximately 66% of the Ca, 33% of the Mg, 50% of the inorganic phosphorus (P_i) and 10% of the citrate (Cit) are bound to casein micelles and thus are non-ultrafilterable (White and Davies, 1958; McGann and Pyne, 1960), but the available evidence indicates that the partition of milk salts in non-bovine milks can be appreciably different. Thus, whereas the total concentrations and the partition of major salts in the milks of the goat (*Capra hircus*) and water buffalo are similar to values for the cow (Abd El-Salam and El-Shibiny, 1966; Albonico *et al.*, 1969; Jenness, 1980), Kondo *et al.* (1964) found that, in 22 samples of human milk, on average about 65% of the casein was sedimented by centrifugation at 105,400 *g* for 20 min, but only about 13% of the Ca and 7% of the P_i were present in the pellet. O'Connor and Fox (1977) compared the partition of salts in cow milk with that in milks of the sheep (*Ovis aries*), goat, horse (*Equus caballus*), ass (*Equus asinus*) and pig (*Sus scrofa*). They used rennet whey to estimate ultrafilterable concentrations and adjusted milk pH to 6.6 before treatment with chymosin so their values are not strictly comparable with those of equilibrium dialysis or ultrafiltration. Nevertheless, they do show that the proportions of colloidal Ca and P_i in horse, ass and pig milks are appreciably different from those in the cow, goat, water buffalo and sheep. For example, in the horse about 56% of the Ca and 65% of the P_i were bound to the clotted casein and the corresponding figures for the pig were about 73% for Ca and 83% for P_i. No data on the partition of Mg and Cit were given by either Kondo *et al.* (1964) or O'Connor and Fox (1977).

The physical principles which govern the partition of milk salts are, at present, incompletely understood. The concentrations of ultrafilterable Ca and Cit are closely correlated in cow and goat milks (Holt and Muir, 1979), probably because a strong, soluble, complex, $CaCit^-$, can be formed at milk pH. Variations in citrate metabolism in the mammary gland and milk citrate concentration can be produced by nutritional, biochemical and physiological factors (Ormrod *et al.*, 1979, 1980; Holt *et al.*, 1980; Faulkner, 1980; Faulkner and Clapperton, 1981; Ormrod *et al.*, 1982). The free or ionised Ca concentration, $[Ca^{2+}]$, is related to pH and the concentration of ultrafilterable P_i, $[P_{i,u}]$, through an equation for the solubility of milk calcium phosphate which takes the form (Holt, 1982):

$$K_s = [Ca^{2+}] \cdot [HPO_4^{2-}]^{0.7} \cdot [PO_4^{3-}]^{0.2} \qquad (1)$$

where K_s is the solubility product at the appropriate ionic strength. Equation (1), which is close to the form expected for a dicalcium phosphate, provides a satisfactory description of natural variations in $[Ca^{2+}]$ and $[P_{i,u}]$ in cow milk ultrafiltrates (Holt, 1982) and of changes in these quantities which occur in goat milk diffusates before, during and following a period of starvation (I. H. L. Ormrod *et al.*, 1982).

The colloidal Ca (Ca_c) of bovine milk is partly a calcium phosphate salt, incorporated in casein micelles, and partly Ca bound directly to the casein

proteins. The X-ray absorption spectrum of bovine milk calcium phosphate, measured near the Ca *K*-edge, indicates that it resembles closely the mineral brushite, $CaHPO_4 \cdot 2H_2O$ (Holt *et al.*, 1982). It is presumed that the Ca bound directly to casein follows a binding isotherm and hence is dependent on $[Ca^{2+}]$. Thus, to a good approximation:

$$[Ca_c] = \bar{\nu}_{Ca}[Cas] + \alpha[P_{ic}] \quad (2)$$

where $\bar{\nu}_{Ca}$ is the number of moles of directly-bound Ca per mole of casein, [Cas] is the molar concentration of casein and α is a constant. An experimental determination of α (Holt, 1982) gave 1.61 ± 0.04 which differs appreciably from the 1.0 of a dicalcium phosphate salt, as suggested by solubility and X-ray absorption studies. The explanation offered (Holt *et al.*, 1982) is that phosphate moieties from phosphoserine and phosphothreonine residues in the casein can substitute for P_i at surface sites in the brushite lattice. Thus, the value for α depends on casein P content as well as on the stoichiometry of the mineral phase.

The partition of Mg in cow milk appears to be qualitatively similar to that of Ca to the extent that part of it is bound directly to casein and part of it is incorporated in the milk calcium phosphate (Holt, 1982). In contrast, colloidal citrate in cow milk is associated entirely with the colloidal calcium phosphate (Pyne and McGann, 1960; Holt, 1982).

The species studied produce milks which span a wide range of salt compositions and one of our objectives was to see if the physical principles which underlie the partition of salts in cow milk are more generally valid. In this paper we give the first detailed analyses of the partitioning of salts in milks of the rat (*Rattus norvegicus*) pig and rabbit (*Oryctolagus cuniculus*), all of which contain much higher levels of Ca and P than cow milk. We also give more-detailed information than has been available hitherto on the partition of salts in the milks of man and horse, which contain much less Ca and P than cow milk, and include new results for goat and sheep milks.

Finally, the osmotic factors which regulate milk secretion are examined in the light of a recent theoretical treatment of the main physiological mechanism of milk secretion (Holt, 1983). It has been recognised for many years that the osmolarity of milk is close to that of blood (Taylor and Husband, 1922) and Gachev (1972) showed negative correlations between [lactose] and [Na] and [K] in 10 species. Further results are given here on the relationship between the osmotic contributions of milk salts and lactose. By considering the consequences of osmotic equilibrium on the synthesis of lactose, it is possible to relate the concentration of casein in skim-milk, to that in the Golgi apparatus of the secretory cell ($[Cas]_m$ and $[Cas]_g$ respectively). Casein is packaged for export from the secretory cell in unswollen vesicles which bud-off the Golgi apparatus. These vesicles then undergo swelling due to the intravesicular synthesis of lactose, coupled to salt transport mechanisms, and a quantitative, theoretical, treatment of this process leads to the relation:

$$[Cas]_m = [Cas]_g\{1-(1+\omega)[L]_m/\pi\} \quad (3)$$

where ω is a parameter characterising the coupling of lactose synthesis to the transport of anions into milk, π is the milk osmolarity and $[L]_m$ is the skim-milk concentration of lactose. Approximations made in the theoretical analysis, together with the possibility of post-secretory changes in milk composition due to paracellular ion and lactose movements, make it difficult to apply equation (3) to the analysis of small, intraspecific, changes in milk composition. Larger, interspecific, differences of concentrations offer a better means of testing its validity. If $[Cas]_g$ can vary only a little and ω is effectively constant, equation (3) predicts a negative correlation of lactose and casein concentrations in skim-milk.

MATERIALS AND METHODS

Milk samples

Rat milk was obtained by gentle hand-milking of 24 primiparous Sprague–Dawley rats at an average of 19 days *post partum*. The animals were separated from their young about 6 hr before milking and anaesthetised with 7.2 mg Diabutal given intraperitoneally. Oxytocin (0.3 U) was administered, also intraperitoneally, to facilitate milk removal. Rabbit milk was obtained from 1 black crossbred rabbit, 19 days *post partum* and 2 New Zealand White rabbits 7 and 13 days *post partum*. Litters were removed 7 hr before hand-milking without anaesthesia but with 5 U of oxytocin administered intraperitoneally. Pig milk was obtained from 2 Landrace pigs in the third week of their second lactation, following the injection of 1.5 U oxytocin into the ear vein. Sheep milk was obtained from 2 Finn-Dorset crossbred sheep in their 4th and 6th week of lactation, by hand-milking without oxytocin. Goat milk was taken from 6 Saanen goats in their 6th week of lactation, again without the use of oxytocin. Human milk was obtained by manual expression from 4 healthy women in the 4th week of lactation. The horse milk sample was taken from a single gland of an Arabian horse, approximately 4 months *post partum*. The foal had been separated from the mare for 3 hr and was allowed to suckle the other gland during milking.

Milk samples from a species were pooled and skimmed within 4 hr of collection and the pH determined by glass electrode. An ultrafiltrate was prepared by pressure filtration at about 80 kPa through a Chemlab G10T membrane (Chemlab Instruments Ltd., Hornchurch, Essex) except for the rat, rabbit and horse milks which were filtered through an Amicon PM10 membrane (Amicon Ltd., Queens Road, High Wycombe) and the pH of the ultrafiltrate recorded. Samples of the skim-milk and ultrafiltrate were stored, in screw-cap polythene bottles, at $-20^{\circ}C$ until required for analysis, except for samples from rat, rabbit and horse which were freeze-dried and stored at $-20^{\circ}C$. The freeze-dried samples were reconstituted with the appropriate volume of distilled water, and frozen samples were thawed rapidly by immersing the container in tepid water. Ultrafiltrates required a drop of concentrated nitric acid to dissolve an otherwise insoluble, small, residue consisting largely of Ca and P_i.

Analytical methods

The methods of analysis were adaptations of ones used in the analysis of bovine milks. Nitrogen was determined by a semi-micro Kjeldahl digestion procedure using a selenium catalyst, and ammonia determined colorimetrically by the salicylate-dichloroisocyanurate reaction, essentially as described by Havilah *et al.* (1977). Casein concentration (in g protein/l skim-milk) was determined as the difference between total nitrogen and non-casein nitrogen content (in g N/l skim-milk) using a conversion factor of 6.39. Non-casein nitrogen was determined after precipitation of the casein at pH 4.6 according to the method of Rowland (1933) with subsequent modifications (see Aschaffenburg and Drewry, 1959). Lactose was determined enzymically according to the method of Kurz

and Wallenfels (1974) and phosphorus by the method of Allen (1940) with modifications to determine total P_t, ultrafilterable P_t and ultrafilterable P as described by White and Davies (1958). Citrate was determined by the pyridine/acetic anhydride method of Marier and Boulet (1958) as modified by White and Davies (1963), and, for the rat milk sample, by an enzymic method in which citrate is converted to lactate and malate (Dagley, 1974; Mutzelburg, 1979). Milk and ultrafiltrate Ca and Mg were precipitated as oxalate and ammonium phosphate salts respectively and determined by atomic absorption spectroscopy; Na and K were determined by flame emmission spectroscopy (Holt *et al.*, 1981).

Calculation of ionic equilibria

The principal cations (K, Na, Ca and Mg) and anions (Cl, Cit, P_i and phosphate esters) of cow milk ultrafiltrate interact in solution to form a number of complexes, the concentrations of which can be calculated with reasonable accuracy (Holt *et al.*, 1981). As a result the ionic strength, the concentrations of free Ca^{2+} and Mg^{2+} and the contribution that diffusible ions and complexes make to the osmolarity of milk can be determined. Not all the anions in cow milk ultrafiltrate are measured because large numbers of minor constituents, are present also, but usually amounting to less than 10% of total anion charge. For the purpose of calculation these are treated as a single group with the association constants of a monocarboxylic acid. This method has been applied to the ultrafiltrates of the 7 skim-milk samples, although as noted below, the minor anion fraction is sometimes more significant than in cow milk.

RESULTS AND DISCUSSION

Comparison with previous data

The analytical results for the ultrafiltrate and skim-milk samples are summarised in Table 1. For the purpose of comparison, the concentrations of the same constituents in cow milk are also given, using the data of White and Davies (1958) for herd bulk milk from Ayrshire cows. As would be expected from the small number of samples studied, the total concentrations of constituents are not typical, in every respect, of the milks produced by each species, but the values are, where this has been determined, generally within the range of intraspecific variation. For example, the goat milk sample contained lower levels than expected of Ca, P_i and casein, but in all other respects was reasonably typical (Parkash and Jenness, 1968; Jenness, 1980). The human milk sample contained lower levels of Na, Cl and lactose and higher levels of casein and K than have been reported as typical (Jenness, 1979). The lower lactose content could result from differences of method, for the enzymic reaction used here is likely to approximate closely the true lactose concentration whereas methods based on reducing power or optical rotation will include contributions from the human milk oligosaccharides (Jenness, 1979a). The high casein content is probably due to the relatively early stage of lactation at which samples were taken. Casein N was 41% of total N which is typical of values for human milk by this method (Jenness, 1979a) although Hambraeus *et al.* (1978) and Nagasawa *et al.* (1974) have obtained evidence that the precipitate at pH 4.6 contains some non-casein proteins. The low Na and Cl and high K concentrations indicate that the donors were free of mastitic infection at the time of sampling (Hanwell and Peaker, 1977; Peaker, 1977; Connor, 1979). The sheep milk sample appears to be typical of that species in all respects (Godden and Puddy, 1935; Perrin, 1958; Konar *et al.*, 1971), as is that of the pig (Perrin, 1955; Salmon-Legagneur, 1961, 1964; Fahmy, 1972) with the exceptions that Ca, P_i and casein are higher than normal (Perrin, 1955; Hoynes and Fox, 1975; Lodge, 1959). Similarly, the Ca content of the rabbit milk appears higher than other reported values which range from 60 to 170 mM (Schley, 1975; Lebas *et al.*, 1971). The samples of horse and rat milks are reasonably typical in composition (Neseni *et al.*, 1958; Sutton *et al.*, 1977; M. Peaker, private communication; Chalk and Bailey, 1978; Keen *et al.*, 1981) except for a low chloride concentration in the rat milk compared to the range of about 50–65 mM reported by Chalk and Bailey (1978). However, Chalk and Bailey (1982) milked the same rats several times during the period of lactation, a procedure which can alter milk composition (Keen *et al.*, 1980), including the raising of Cl concentration.

The paucity of literature data on the partition of salts in non-bovine milks makes it impossible to establish whether the diffusible concentrations in Table 1 are representative. The diffusible Ca and P_i concentrations in cow, goat, sheep, horse and pig milks are closer to the values reported by O'Connor and Fox (1977) than are the corresponding total concentrations. Moreover, the generally smaller values in the

Table 1. Concentrations of total and ultrafilterable salts, lactose and casein in skim-milk samples from eight species (concentrations in m mol/litre except casein in g/litre)

	Rat	Rabbit	Pig	Sheep	Goat	Horse	Man	Cow*
Ca_t	80.4	214.4	104.1	56.8	23.1	16.5	7.8	29.4
Ca_u	2.5	14.0	7.1	5.2	8.0	6.4	5.0	9.2
Mg_t	8.8	19.5	9.6	9.0	5.0	1.6	1.1	5.1
Mg_u	1.0	7.3	1.9	2.9	3.4	1.3	1.0	3.3
$P_{i,t}$	54.2	93.3	51.2	39.7	15.6	6.7	2.5	20.9
$P_{i,u}$	12.2	6.7	3.3	11.7	9.0	4.1	1.9	11.2
EP_u	10.0	26.4	11.1	12.9	8.7	3.3	2.7	3.5
Cit_t	0.06	17.4	8.9	4.9	5.4	3.1	2.8	9.2
Cit_u	0.04	9.1	3.0	3.8	5.0	3.1	2.7	8.2
Na_u	38.3	83.7	14.4	20.5	20.5	5.7	5.0	24.2
K_u	43.6	89.5	31.4	31.7	46.6	11.9	16.5	34.7
Cl_u	36.1	80	28.7	17.0	34.2	6.6	6.2	30.2
Casein	70.0	104	41.4	58.0	18.9	8.2	8.8	26.1
Lactose	112	59.1	167	147	123	222	194	137

*Herd bulk milk data of White and Davies (1958).
EP, phosphate esters.

Table 2. Ion equilibria parameters in milk ultrafiltrates from eight species

	Rat	Rabbit	Pig	Sheep	Goat	Horse	Man	Cow
pH	6.90	7.25*	7.00	6.88	6.66	7.00	7.22	6.72
Ca^{2+}	1.3	3.8	2.8	1.4	2.6	2.5	1.9	2.00
Mg^{2+}	0.5	2.1	0.8	0.8	1.2	0.6	0.4	0.81
Salt osmolarity	113	230	71	72	105	34	39	94
Ionic strength	90	200	63	70	84	30	31	73
Saturation index	0.60	1.04	0.60	0.77	0.70	0.91	0.48	0.84

*pH of ultrafiltrate not measured but assumed equal to that of the skim-milk.
Concentrations are mM.

present work could result in part from the use by O'Connor and Fox (1977) of a rennet whey and a pH adjustment which will overestimate diffusible Ca and P_i concentrations (Davies and White, 1960; Pyne and McGann, 1960). In the experiments of Kondo *et al.* (1964) on human milk, about 65% of the casein was sedimented along with 0.9 mM Ca and 0.23 mM P_i. These values undoubtedly underestimate colloidal concentrations and are less, by factors of 3.1 and 2.6 respectively, than the values for human milk obtained from Table 1. It appears from these results that a considerable fraction of the colloidal Ca and P_i in human milk may be bound to relatively low molecular weight material.

Ion equilibria calculations

The measured ultrafiltrate concentrations allow some approximate calculations to be made of the concentrations of free ions and complexes. These calculations are subject to a greater uncertainty than are those for cow milk for two reasons. First, the phosphate ester fraction is of greater importance but the identity and equilibrium constants of these components are largely unknown: it is assumed that they are equivalent to glucose-1-phosphate in their ion-binding properties. Second, a carboxylic acid fraction is introduced to achieve charge balance in the ultrafiltrate because the chemical analysis did not include quantitative determination of the very many minor anion constituents (Holt *et al.*, 1981). Results of the calculations, shown in Table 2, include the free ion concentrations $[Ca^{2+}]$ and $[Mg^{2+}]$, the calculated ionic strength, the contribution of diffusible salts to the milk osmolarity and a saturation index. The latter is an ion activity product divided by the corresponding solubility product (equation 1) which, in cow milk, has no dependence on pH (Holt, 1982). It can be seen that the free ion concentrations of Ca and Mg are much less than diffusible concentrations because of complex formation and are generally similar to those in cow milk. A calcium ion concentration of a few mM is required to prevent dissociation of casein from micelles and the free magnesium ion concentrations are of the same order as blood and cell cytosol levels. Saturation indices range from 0.48 in the human milk to 1.04 in that of the rabbit but like the free ion concentrations, these values are subject to an error of unknown magnitude because of the phosphate ester and carboxylic acid fractions. All the milk diffusates were supersaturated with respect to brushite, the mineral with a short-range structure which resembles most closely that of the milk calcium phosphate (Holt *et al.*, 1982). Hence the dissolution of the mineral phase, with a consequent dissociation of caseins from the micelles, is prevented. Given that, for reasons of micelle stability, ultrafiltrates should have a minimum free Ca ion concentration and be saturated or supersaturated in calcium phosphate, a constraint is placed on the range of possible compositions of ultrafiltrates. In particular, since P_i is largely present as HPO_4^{2-} and $H_2PO_4^-$ ions there is a tendency for ultrafiltrate P_i concentration to be negatively correlated with pH (Fig. 1) as noted by White and Davies (1958) for the cow and by O'Connor and Fox (1977) for a number of other species. The relationship would be more exact if the free Ca ion concentration were constant as can be appreciated by inspection of equation (1).

A second consequence of the ionic requirements for micelle stability is that ultrafiltrate concentrations of Ca and Cit are nearly proportional to one another, as has been demonstrated in the cow (Holt and Muir, 1979) and goat (Ormrod *et al.*, 1982) and is also apparent in Fig. 2. The relationship is, again, more nearly exact at constant free Ca ion concentration. Also shown in Fig. 2 is the proportionality of $[Mg_s]$ to $[Cit_s]$ and, in consequence, the proportionality of $[Mg_s]+[Ca_s]$ to $[Cit_s]$. It should be noted however that in the pig and rabbit milk samples a considerable proportion of total milk citrate is in the colloidal phase and the relationships shown in Fig. 2 apply only to

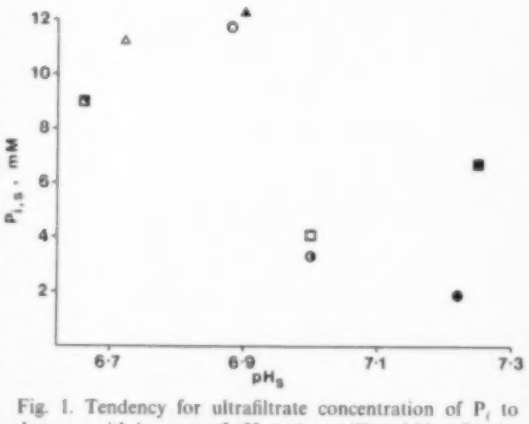

Fig. 1. Tendency for ultrafiltrate concentration of P_i to decrease with increase of pH: (▲) rat; (■) rabbit; (◑) pig; (○) sheep; (◥) goat; (□) horse; (●) man; (△) cow.

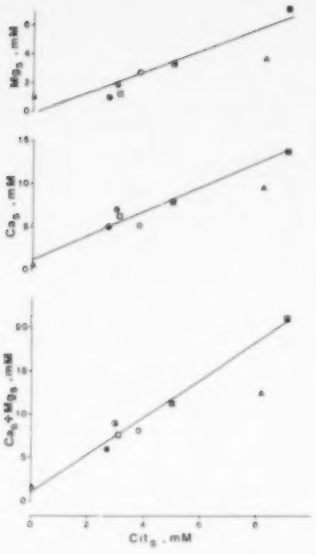

Fig. 2. Correlations of ultrafiltrate concentrations of Ca and Mg with citrate. The least squares regression equations are (a) for all the results: $[Mg_s] = 0.15 \pm 0.76 + 0.60 \pm 0.15\,[Cit_s]$, $r^2 = 0.735$; $[Ca_s] = 2.54 \pm 0.92 + 1.06 \pm 0.18\,[Cit_s]$, $r^2 = 0.858$; $[Ca_s] + [Mg_s] = 3.12 \pm 1.57 + 1.62 \pm 0.30\,[Cit_s]$, $r^2 = 0.827$; and (b) excluding data for the cow: $[Mg_s] = -0.23 \pm 0.55 + 0.76 \pm 0.12\,[Cit_s]$, $r^2 = 0.891$; $[Ca_s] = 2.09 \pm 0.69 + 1.26 \pm 0.15\,[Cit_s]$, $r^2 = 0.933$; $[Ca_s] + [Mg_s] = 2.26 \pm 0.99 + 1.99 \pm 0.22\,[Cit_s]$, $r^2 = 0.944$. Lines shown in the figure correspond to (b). For symbols see Fig. 1.

ultrafilterable components, albeit over a much broader range of concentrations than is accessible through ruminant milks alone.

Osmotic regulation of milk composition

The osmolarity of milk may be represented as the sum of the osmotic contributions of lactose, diffusible salts (S) and other, uncharged, components (U). Neglecting activity coefficients for uncharged species:

$$\pi = [L] + [U] + \bar{\gamma}[S] \qquad (4)$$

where $\bar{\gamma}$ is the mean ion activity coefficient of the diffusible salts. The sum of the activities of diffusible ions and complexes is readily obtained from the ion equilibria calculations described above so provided [U] remains small compared to π, or constant, [L] should be negatively correlated with the salt osmolarity if total osmolarity is the same for each of the species studied. For seven of the eight species the results in Fig. 3 give $\pi - [U] = 252$ mM or $[U] \sim 50$ mM if $\pi \sim 300$ mM. The result for the rabbit milk does not conform to the general relationship, presumably because of a low content of components of the U category.

Colloidal concentrations

If there are no appreciable post-secretory changes in milk composition, the concentration of casein may be related to that of lactose by equation (3). With the exception of the goat and cow milk samples, which have lower casein concentrations than expected, results fall on a straight line (Fig. 4) demonstrating a negative correlation of casein and lactose concentrations in the skim-milks. The intercept at zero lactose concentration gives the casein concentration in the Golgi apparatus (141.9 ± 9.6 g/l skim-milk), a concentration which, since casein micelles have not been formed at this stage of secretion, will give a fairly viscous solution. It may be of interest to note that in a number of species of seals and whales the protein concentration is about 100 g/l skim-milk and since the milks contain very little carbohydrate (e.g. Riedman and Ortiz, 1979) only a small degree of osmotic dilution of the Golgi apparatus concentration may be expected (Holt, 1983). Under such a circumstance the milk protein concentration should be, as it is, of the same order as that in the Golgi apparatus. From the intercept and slope of the fitted line, $1 + \omega$ is calculated to be 1.32 ± 0.16 which is comparable to the value 1.50 calculated from the results of Rook and Campling (1965) on the compositional changes in cow milk during the first 5 days of lactation (Holt, 1983). The

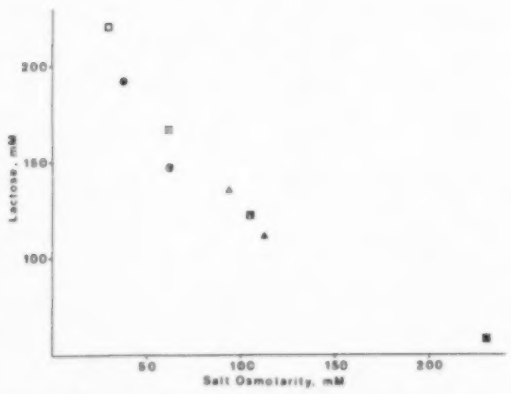

Fig. 3. Variation of lactose concentration with salt osmolarity in skim-milk. For symbols see Fig. 1.

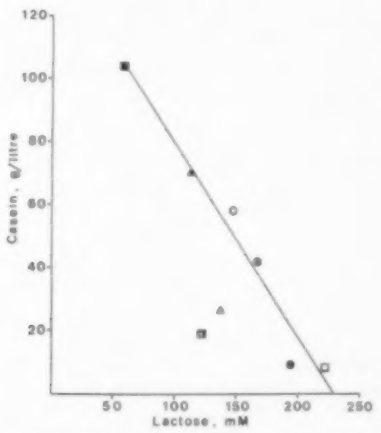

Fig. 4. Relationship between casein and lactose concentrations in skim-milks. Excluding results for the goat and data for the cow (see text) the calculated least squares regression equation is: $[Cas] = 141.9 \pm 9.6 - 0.622 \pm 0.061\,[L]$; $r^2 = 0.964$. For symbols see Fig. 1.

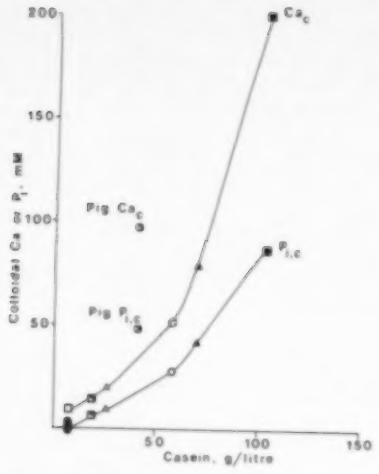

Fig. 5. Variation in colloidal Ca and P_i concentrations with that of casein in the skim-milk samples. For symbols see Fig. 1.

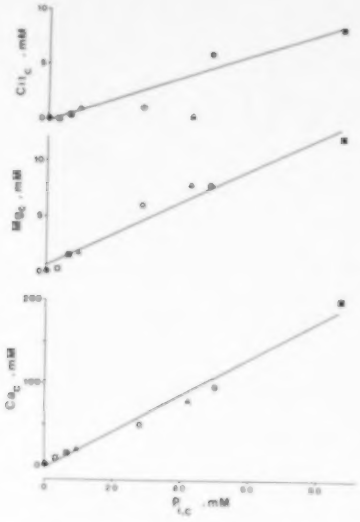

Fig. 6. Dependence of the colloidal concentrations of Ca, Mg and citrate on the colloidal P_i concentration. Calculated least squares regression equations are: $[Ca_c] = 2.4 \pm 4.2 + 2.21 \pm 0.11$ $[P_{i,c}]$, $r^2 = 0.986$; $[Mg_c] = 0.64 \pm 0.46 + 0.145 \pm 0.012$ $[P_{i,c}]$, $r^2 = 0.963$; $[Cit_c] = -0.24 \pm 0.46 + 0.101 \pm 0.012$ $[P_{i,c}]$; $r^2 = 0.938$. In the equation for colloidal citrate, the result for the rat was excluded from the regression since this milk sample contained virtually no citrate. For symbols see Fig. 1.

intercept of the fitted line at zero casein concentration shows that the maximum possible lactose concentration is about 230 mM, which is comparable to lactose concentrations in the milks of man and some other primates, and the horse (Jenness, 1974). Thus the full range of compositions that can be produced by a simple secretory mechanism involving the swelling of Golgi vesicles are represented within the Mammalia. It may be argued, therefore, that variation in the degree of swelling of Golgi vesicles prior to exocytosis constitutes the main source of interspecific variation in the concentrations of milk constituents. A lesser source of variation could be the casein concentration in the Golgi apparatus and unswollen vesicle, established by the balance of synthesis rate of protein with export in Golgi vesicles. A reduction in this quantity could be responsible for the observation that casein concentrations in cow and goat milks are lower than expected from their lactose concentrations.

The concentrations of colloidal Ca and P_i vary with the concentration of casein (Fig. 5). Jenness (1979b) earlier pointed out the correlation of total Ca and total P with casein and these are usually due to the predominant contribution of micellar Ca and P_i to the total concentrations. The amounts of micellar Ca, Mg and P_i per unit weight of casein increase as casein concentration rises although the pig milk sample deviates from the general trend in having higher colloidal Ca and P_i concentrations than expected from its casein content. As noted above, the pig milk sample was not typical of that species; it contained total Ca and P_i concentrations above the normal range.

Interspecific differences in calcium phosphate are an order of magnitude larger than differences of diffusible Ca associated with diffusible citrate, aad the major source of the differences in milk Ca content. Colloidal Ca and Mg exhibit a significant positive correlation with colloidal P_i (Fig. 6).

Concentration and distribution of citrate

Citrate is synthesized within the mitochondria of the secretory cells and passes thence into the cytosol (Faulkner and Peaker, 1982). There it is subject to cleavage by ATP-citrate lyase to oxaloacetate and acetyl CoA—and, after isomerization to isocitrate, oxidation to 2-oxoglutarate with the formation of NADPH. It can enter the Golgi apparatus, perhaps by an active transport mechanism (see Faulkner and Peaker, 1982) and once there may be incorporated into the calcium phosphate of casein micelles. The final concentration of citrate in milk and its distribution between the ultrafilterable and micellar states is thus the result of the relative rates of synthesis, cleavage, oxidation, transport into the Golgi vesicles and binding in the micelles. Rat mammary gland has a very high activity of ATP-citrate lyase (Jenness and Sloan, 1970) and evidently citrate is largely cleaved with the result that little is secreted in the milk (Table 1). Of the other 7 species goat, cow and rabbit have very low mammary ATP-citrate lyase activities and pig has an intermediate level (Baldwin, 1966); data are lacking for horse and man. The distribution of citrate and to a considerable extent the total concentration as well is determined by the extent of binding in the micelles. Figure 6 shows that for our seven species other than rat, colloidal citrate is closely correlated with colloidal P_i. Thus rabbit and cow milks with low ATP-citrate lyase have similar contents of Cit_t but the former has much more Cit_c. Pig milk with intermediate ATP-citrate lyase has lower Cit_t than cow or rabbit but much more Cit_c than cow because of its much larger concentration of colloidal phosphate in the micelles.

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EFFECTS OF BACTERIAL CONTAMINATION ON DEVELOPMENT AND BLOOD CHEMISTRY OF *HELIOTHIS VIRESCENS*

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(*Received* 4 *May* 1983)

Abstract—1. The age of *Heliothis virescens* (F.) larvae at the time of bacterial contamination of their diet, as well as contaminant species, had a significant influence on yield of the pupae.

2. Pupae produced on contaminated diet weighed less than normal.

3. All species of contaminating bacteria studied had detrimental effects on egg hatch.

4. The carbohydrate level in larvae contaminated by *Bacillus subtilis* (Ehrenberg) Cohn was significantly less than that of the control and that of larvae exposed to *Escherichia coli* (Migula) Castellani and Chalmers, and *Staphylococcus epidermidis* (Winslow and Winslow) Evans.

5. The significant decrease of uric acid detected in the blood of *H. virescens* contaminated with *B. subtilis* and *S. epidermidis* may be indicative of increased excretion of water in the feces.

6. There was an unexplained significant increase of cholesterol in the blood of *S. epidermidis* treated larvae.

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INTRODUCTION

Insectary-reared insects often are contaminated by various species of bacteria, which may have harmful effects on the insects' development and vigor. Infection of lepidopterous larvae with microbial contaminants caused high mortality and delay in larval development according to Bell *et al.* (1981); F. Davis (unpublished, U.S. Department of Agriculture, P.O. Box 5367, Mississippi State, MS 39762); G. A. Virginio (unpublished, Diga International Company, 9233 Colorado Street, Riverside, CA 92502); and others. They also observed that infections which did not kill the larvae resulted in diminutive pupae and adults. Maeda *et al.* (1953) reported on effects of microbial contamination on *Dacus dorsalis* Hendel.

The impact of microbial contamination on rearing of boll weevils (*Anthonomus grandis* Boheman) has been documented by Gast (1966), Gast and Davich (1966), McLaughlin (1970), Sikorowski (1975), Sikorowski *et al.* (1977), Gueldner *et al.* (1977), Thompson *et al.* (1977) and Sikorowski and Thompson (1980). It is difficult, if not impossible, to rear high-quality insects in an insectary with a high incidence of microbial diet contamination and consequent insect diseases.

This paper deals with effects of bacterial contamination on (1) development, (2) blood chemistry, and (3) content of protein and carbohydrates in the larvae.

MATERIALS AND METHODS

Source of insects and inoculum

Tobacco budworm, *Heliothis virescens* F., used in the tests were of laboratory stock reared on artificial medium (Berger, 1963) and maintained as described by Thompson and Sikorowski (1983). *Bacillus subtilis* (Ehrenberg) Cohn and *Escherichia coli* (Migula) Castellani and Chalmers were obtained from The American Type Culture Collection. *Staphylococcus epidermidis* (Winslow and Winslow) Evans was our own isolate. All three bacterial species were reported as insect media or insect contaminates. Bacteria were cultivated on trypticase soy agar or *Heliothis* diet free of antimicrobials.

Treatment

Two-, six- or nine-day-old larvae were transferred from bacteria-free to bacterially-contaminated diet. The treated larvae were reared individually in 30-ml plastic cups and incubated at 25 ± 2°C. Untreated larvae were used as controls. The number of pupae that originated from each group of larvae was recorded. For determination of the effects of bacterial contamination on weight of pupae, adult emergence, egg hatch and hemolymph chemistry, contaminated diet was fed to nine-day-old larvae until pupation.

Protein and carbohydrate analysis

Total protein was determined according to the method of Bradford (1976) on five insects for each species of bacteria. Carbohydrates were quantified by the method of Carrol *et al.* (1956).

Blood chemistry

Droplets of hemolymph issuing from the wounds caused by removal of prolegs were collected in 100 μl micro-pipettes. Each sample of 100μl of blood represented a pooled sample from 20 larvae and each test was repeated four times. For determination of sodium, both flame photometry and atomic absorption methods were used. There was no significant difference between the results obtained with either method. Results obtained with flame photometry were used in the study. Blood components were identified and quantitated by the Toxicology Department, Southern Research Institute, Birmingham, AL 35255.

RESULTS AND DISCUSSION

The effects on pupation of contaminated *H. virescens* diet are given in Table 1. The age of the larvae at time of diet contamination (and subsequent

Table 1. Effects of bacterial contamination on pupation of *Heliothis virescens*•

Contaminant	♀ (%) pupated Day contaminated (4 rep; 50 larvae/rep) 2	6	9
Bacillus subtilis	0.0a	4.5a	35.0b
Staphylococcus epidermidis	34.0b	32.0b	49.5b
Escherichia coli	33.5b	40.0b	50.5b
Control	90.5c	90.5c	90.5c

•Means followed by the same letter vertically are not significantly different at $P = 0.10$ according to the Duncan Multiple Range Test.

Table 2. Effects of bacterial contamination on development of *Heliothis virescens*-contaminated diet fed to 9-day-old larvae• (4 rep; 50 pupae/rep)

Bacteria used	Weight of pupae (g)	Adult emergence (%)	Egg hatch (%)
Bacillus subtilis	0.2232a	27.31a	62.32a
Staphylococcus epidermidis	0.2832b	55.23b	63.23a
Escherichia coli	0.2934b	58.67b	62.53a
Control	0.3204c	65.44b	72.01b

•Means within a column not followed by the same letter differ significantly at the 5% level of probability according to Duncan's Multiple Range Test (4 rep; 50 pupae/rep).

Table 3. Protein and carbohydrates of healthy and bacterially contaminated 9-day-old *Heliothis virescens* larvae

	Concentration Protein (mg/Insect)	Carbohydrates (Cal/Insect)
Check	34.0 ± 2.4	32.8 ± 5.2
B. subtilis	30.4 ± 6.8	20.1 ± 4.8
E. coli	39.6 ± 6.9	32.8 ± 3.7
S. epidermidis	35.7 ± 3.4	40.4 ± 8.2

contamination of the larvae), as well as the species contaminating the diet, have significant influence on yield of the pupae. The insect is very susceptible to *B. subtilis* contamination during the larval stage. The effects of contamination on the larvae decrease with age of larvae (Table 1).

The effects on development of tobacco budworm caused by feeding contaminated diet to nine-day-old larvae are given in Table 2. Pupae produced on contaminated diet weighed less than normal. Pupae developed from *S. epidermidis*- and *E. coli*-contaminated larvae produced equal numbers of adults to that of the control, but from *B. subtilis* the number of adults was significantly reduced. All species of bacteria caused a decrease in egg hatch.

The effects of microbial contamination on protein of *H. virescens* were not significantly different from the control (Table 3). However, the carbohydrate level of *B. subtilis*-contaminated larvae was significantly decreased compared to the control and compared to larvae exposed to other bacterial contaminants. Previously we have shown (Thompson and Sikorowski, 1980) that in *H. virescens*, carbohydrate (glycogen) is the principal source of energy for emergence from pupae to adults. This interference of *B. subtilis* in carbohydrates accumulation (Table 3) may explain some of the effects noted in Tables 1 and 2.

Clinical chemical analyses on the hemolymph from field, laboratory-reared and bacterially-contaminated *H. virescens* larvae are given in Table 4. Some values of blood constituents appear to be unaffected by contamination—for example calcium, sodium, potassium, albumin—whereas others—cholesterol and uric acid—are significantly altered by them.

Although chloride ion concentration in the blood of *S. epidermidis*-contaminated *H. virescens* is significantly different from that of field-collected larvae, it falls within the normal range for phytophagous insects (Buck, 1953).

We are unable to explain the significant increase of cholesterol in the blood of *S. epidermidis*-treated larvae when compared to other treatments. Pupal develop-

Table 4. Clinical chemistry analysis on hemolymph from bacteri-contaminated *Heliothis virescens* larvae•

Test	Reared on microbially contaminated diet *B. subtilis*	*E. coli*	*S. epidermidis*	Reared on bacteria-free diet	Collected in the cotton field
Albumin (g/dl)	1.57a	2.03a	2.06a	1.70a	1.10a
BUN (mg/dl)	28.02a	33.05a	23.52a	22.02a	9.73a
Calcium (mg/dl)	27.79a	31.51a	28.91a	28.41a	28.85a
Chloride (meq/l)	103.45ab	82.99ab	121.94 b	92.45ab	48.55a
Cholesterol eq (mg/dl)	37.10a	45.72a	101.90 b	46.74a	96.10ab
Glucose (mg/dl)	71.10a	73.19a	69.64a	69.82a	28.60a
Magnesium (mg/dl)	26.78a	24.77a	22.95a	26.79a	89.79 b
Phosphorous (mg/dl)	16.84 b	19.17 b	18.32 b	17.39 b	6.00a
Phosphorous (mg/dl)†	16.84ab	19.17 b	18.32ab	17.39ab	6.00a
Potassium (meq/l)	38.19a	39.27a	37.11a	37.45a	41.65a
Sodium (meq/l)	15.27a	13.17a	13.22a	13.27a	–
Bilirubin (mg/dl)	7.08a	6.80a	7.30a	7.32a	4.13a
Protein (g/dl)	4.12ab	4.94ab	5.57 b	4.46ab	3.27a
Protein (g/dl)†	4.12ab	4.94ab	5.57 b	4.46ab	3.27a
Triglycerides (mg/dl)	471.23a	536.03a	473.33a	410.87a	485.30a
Uric Acid (mg/dl)	33.40a	48.15ab	70.56 b	44.76ab	43.15ab

•Means followed by the same letter horizontally are not significantly different at $P = 0.10$ according to the Duncan Multiple Range Test.
†Significantly different also at $P = 0.05$.

ment and numbers of adults produced by *H. virescens* fed this contaminated diet are equal to that of the control (Table 1). The accumulation of the sterol may be a function of the bacteria, without any ill effects on the host.

Although magnesium ion concentration is significantly lower in the blood of diet fed insects compared to plant-fed insects, the growth and length of time of ecdysis is not extended. We have observed (unpublished) that ecdysis in *H. virescens* is shortened by as much as five days on larval diet from that of larvae fed on sweet clover (one of the host plants).

Any observed differences in *H. virescens* larvae fed the bacterially-treated diet and those on the control diet did not result in an imbalance of the Na/K ratio as observed for the boll weevil, *Anthonomus grandis*, when sterilized by chemicals or radiation (Thompson and Sikorowski, 1982). Thus, the integrity of the genetic character of the host is not affected by these bacteria since the constancy of the Na/K ratio is genetically controlled (Buck, 1953).

The significant decrease of uric acid in the blood of *H. virescens* contaminated with *B. subtilis* and *S. epidermidis* (Table 4) may be indicative of an increased excretion of water in the feces. Since water retention is associated with nitrogen detoxification, the weight loss observed for the contaminated insects (Table 1) may be due in part to a loss of water.

In summary, the *Heliothis* diet may provide growth medium for many different microorganisms and these may have varying effects on larval development, weight of pupae, adult emergence, egg hatch, and chemistry of haemolymph. Scientists need laboratory-reared insects of uniformly high quality for research, pest management, etc. Unfortunately, laboratory conditions often facilitate the growth of a wide range of microbial contaminants, which in turn may produce insects of reduced quality unsuited for most, it not all, entomological work as illustrated in this paper.

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ORIGINS OF THE TRANSIENT ANTERIOR–POSTERIOR ASYMMETRY IN THE FROG LENS FIBER POTENTIAL

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Abstract—1. The origin of the transient asymmetry of intracellular resting potentials between the anterior and posterior lens fibers was investigated in the isolated American bullfrog lens by a conventional microelectrode technique.

2. In high K^+, Rb^+, Cs^+, or NH_4^+ test solution applied only to the lens anterior or posterior side, anterior fibers depolarized at a slower rate than posterior ones. After a long exposure, however, the transient potential difference disappeared. The magnitude of the depolarizations of the lens fibers was in the order of $K^+ > Rb^+ > Cs^+ > NH_4^+$.

3. The resting potentials plotted as a function of external K^+ concentrations ($[K]_o$) were in agreement with Nernst equation predictions with a slope of 58 mV/decade ion concentration change.

4. A small Na^+ permeability is unmasked at a $[K]_o$ less than 10 mM.

5. It was concluded that the transient difference measured in potentials of anterior and posterior lens fibers on increasing external K^+, Rb^+, Cs^+ or NH_4^+ depends on the anterior epithelial cell layer, which is a diffusional barrier for ions penetrating into the lens interior.

INTRODUCTION

Asymmetry exists anatomically and functionally in the characteristics of individual lens fibers from the anterior and posterior surfaces on the vertebrate lens. The epithelium consisting of a layer of cells on the lens anterior appears responsible for active Na–K transport mechanism of the lens and influences lens transparency (Bonting *et al.*, 1963; Kinsey and Reddy, 1965). No such active pumping system is found on the posterior surface where an epithelium is absent. Similarly, a few epithelial cell layers in frog and rabbit corneas response to active transport mechanism but also act as a tight barrier against the diffusion of cations, sucrose and metabolic inhibitors from the tear side to the corneal basal cell (Green, 1969; Akaike and Hori, 1970; Akaike, 1971; Akaike and Kiyohara, 1977).

Kinsey and McGrady (1971) showed that the fiber-epithelial potential (inside to outside of lens) and fiber potential of rabbit lens were affected in bathing medium with K^+, Rb^+, or Cs^+ substituted for equivalent concentrations of Na^+. Murata *et al.* (1974) also observed a considerable depolarization of the lens fibers in the isolated frog lens immersed in artificial solution containing 90 mM K^+, Rb^+ or Cs^+. Recently, Delamere and Duncan (1979) have shown that on increasing the bathing K^+ concentration the half-time of the potential change in bovine lens, which was mounted in a divided chamber, was faster in the posterior fiber than in the anterior fiber.

Therefore, the aim of this study is to know whether a layer of epithelial cells on the anterior surface of frog lens affects the rate of diffusion of K^+, Rb^+ or Cs^+ from the anterior side to the lens interior and makes delay on the time courses of the depolarization of fibers at anterior side.

MATERIALS AND METHODS

Dissection and mounting

American bullfrogs (*Rana catesbeiana*) weighing between 250 and 400 g were pithed and the eyes removed. The lens was dissected free of surrounding tissues under dissection microscope. Care was taken not to damage the lens. In order to superfuse separately each surface of a lens by the same or different test solution, the complete isolated *in vitro* lens preparation was mounted in a special glass chamber with the anterior or posterior surface facing up and fixed to the glass cup covered with siliconized cloth and held in place by subatmospheric pressure of between −100 and −130 mmHg on the opposite surface (Fig. 1A and B). Tests by dye solution showed than no solution leaked from one side to the other, though there is the presence of short circuit between the two bathing solutions through pathways such as the lens capsule, because of no translens potential which could be recorded as the anterior surface of lens was electrically isolated from posterior by a divided chamber (Candia *et al.*, 1970; Kinsey and McGrady, 1971; Duncan *et al.*, 1977; Delamere and Duncan, 1979; Akaike and Okajima, 1982). However, the purpose of this study is confirmed enough by the prevention of solution leakage from one side to the other. In fact, in the present experiments, neither anterior nor posterior lens fiber potential was affected by high K^+ test solution added to the opposite side of the potential measurements (see text in detail).

Solutions

Ionic composition of test solution is shown in Table 1. Modified Ringer containing 90 mM K^+, Rb^+, Cs^+ or NH_4^+ was prepared replacing NaCl by an equivalent amount of KCl, RbCl, CsCl or NH_4Cl, respectively. The changes of external K^+ concentrations were made by substituting KCl for NaCl in 120 mM Cl^- Ringer on a mole-to-mole basis (Table 1). The change of bathing medium was accomplished fairly abruptly by switching from one to another. Solution changes were complete within 30 sec since flow rates were adjusted. The test solution, then, was constantly flowing

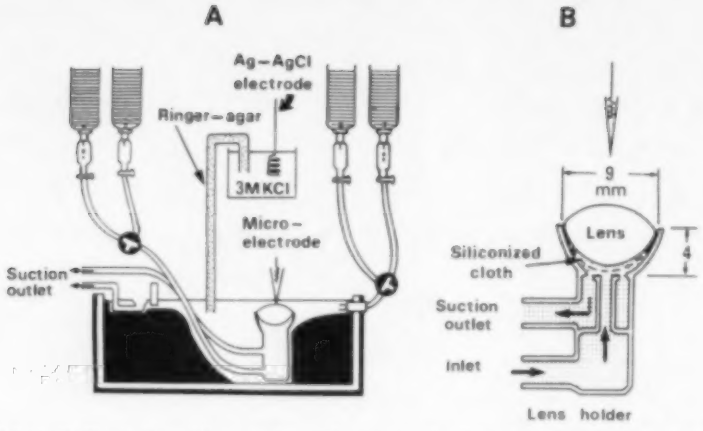

Fig. 1. (A) Schematic illustration of the experimental apparatus for the potential measurements of crystalline lens fibers of isolated bullfrog lens. (B) Glass chamber employed to selective isolated either the anterior or posterior surface of the lens permitting independent perfusion of either surface with same or different artificial test solutions simultaneously.

through each chamber. The bathing solutions were always equilibrated with 95% O_2 and 5% CO_2 throughout the experiments. The pH of all solutions was maintained between 7.2 and 7.4 at room temperature, and any deviation from this pH was adjusted by adding Hepes and Tris-base. The osmolality of test solutions was about 230 mOsmol/l.

Electrophysiological measurements

The intracellular membrane potentials of individual lens fibers in anterior and posterior sides were measured with glass microelectrode filled with 3 M KCl and having tip resistances in the range 20–40 MΩ. The reference electrode was a calomel electrode immersed in 3 M KCl solution which was contacted via a Ringer-agar bridge with bathing medium. The microelectrodes with tip potentials of less than 5 mV in Ringer solution were selected for use. The potential difference between a microelectrode and a reference electrode was displayed on an oscilloscope through d.c. amplifier (Model 750, W.P.I. Instruments Inc.) and recorded continuously with a pen-writing recorder. The first electrical measurements were made at 60 min after the dissection.

The microelectrode was advanced perpendicularly to the plane of lens equator through the anterior or posterior surface into the lens interior by a stepping pulse-motor driven micromanipulator (Model SM-23, Narishige, Japan) under a dissecting microscope. In all experiments, the tip of the microelectrode was inserted to a depth of 200 μm from each lens surface since, in the topographical aspects of frog lens potential, the penetration of microelectrode to a depth of 100–200 μm gave a maximum membrane potential between −90 and −100 mV (Taura *et al.*, 1979).

The numerical values are given as mean value ± 1 SD of the mean. Significance of the difference of means was decided by a Student's *t*-test.

RESULTS

Effects of K^+ and K^+-like cations on the membrane potentials of anterior and posterior lens fibers

In the muscle fibers of barnacle (Hagiwara *et al.*, 1964) and of lobster (Gainer and Grundfest, 1968), and in the corneal epithelial cells of frog and rabbit (Akaike and Hori, 1970; Akaike, 1971), the relative permeability of cell membrane for monovalent cations is in the order of $K^+ > Rb^+ > Cs^+ > NH_4^+$. A systematic investigation has thus been undertaken to correlate potential and ionic environment of lens in order to characterize the nature of the permeability across both the anterior and posterior lens surfaces or/and the lens fiber membrane itself. In the following description, the intracellular membrane potentials of lens fibers were measured at a depth of 200 μm from the anterior or posterior surface, and referred to as "anterior" and "posterior" potentials, respectively.

When only anterior surface of the isolated lens mounted in a glass chamber was bathed with a test Ringer containing 90 mM Rb^+, the anterior lens fibers depolarized from −93.5 ± 3.6 mV (25 fibers from 8 lenses) in control to −23.5 ± 4.7 mV (20 fibers from 6 lenses) within the first 5 min after the solution change. On application of same test solution to the posterior side, the posterior fibers depolarized from −94.0 ± 3.9 mV (18 fibers from 8 lenses) in control to −13.5 ± 3.8 mV (25 fibers from 7 lenses). The depolarization was faster in posterior lens fibers than in anterior ones (Fig. 2A and B) and the difference in depolarized fiber

Table 1. Composition of modified Ringer solution (mM)

Solution	Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-	HCO_3^-	SO_4^-	HPO_4^{2-}	Gluconate	Glucose
Normal Ringer	104.6	2.5	1	1.2	74.7	26	1.2	2.9	2	26
90 mM K^+	16.5	90	1	1.2	90	10.7	1.2	2.9	2	26
90 mM Rb^+	16.5	90 Rb^+	1	1.2	90	10.7	1.2	2.9	2	26
90 mM Cs^+	16.5	90 Cs^+	1	1.2	90	10.7	1.2	2.9	2	26
90 mM NH_4^+	16.5	90 NH_4^+	1	1.2	90	10.7	1.2	2.9	2	26
120 mM Cl^- Ringer	106.7	2.5	1	1.2	120	5	1.2	2.9	2	8

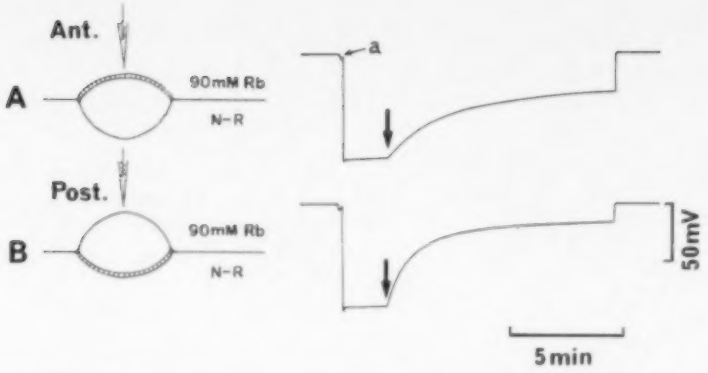

Fig. 2. Depolarizations of the anterior and posterior lens fibers by an application of 90 mM Rb^+. The high Rb^+ test solution was applied to the anterior side for recordings A and to the posterior side for recording B. The preparation was recovered in normal Ringer after the exposure to the high Rb^+ test solution for 10 min. This set of representative recordings were obtained from the same lens. The symbol a in the recording A is the small potential shifts produced by the initial contact of the microelectrode with surface capsule. Maximal intracellular potentials of single lens fibers were encountered at a depth of 200 μm from the lens surface. Arrows show the external solution change.

potentials between the both sides was statistically significant ($P < 0.001$) at 5 min after the exposure to test solution. This difference, however, disappeared at about 100 min after application of test solution (Table 2). No difference remained in the membrane potentials between the anterior and posterior lens fibers. Thereafter, these maximal depolarized potentials were maintained constantly for longer than 3 hr at least. The time taken for half depolarization ($t_{1/2}$) after the application of test solution with 90 mM K^+, Rb^+, Cs^+ or NH_4^+ is summarized in Table 2.

Figures 3 and 4 show the time courses of the changes of lens potential in the lens anterior or posterior side which was immersed in one of test solutions containing 90 mM K^+, Rb^+, Cs^+ and NH_4^+. It is evident in these figures that the rate of depolarization of lens fiber was much more rapid in posterior fibers at early period after exposure to all test solutions. As the anterior and posterior lens fibers were soaked independently in 90 mM K^+, Rb^+, Cs^+ or NH_4^+ test solution for 100 min, the final depolarized potentials at both sides were quite same to each other. In addition, the permselectivities of lens fiber membrane for four monovalent cations were in the same order of $K^+ > Rb^+ > Cs^+ > NH_4^+$ at each side of lens.

The role of lens capsule on the ionic diffusion across the lens surface

Since the anterior lens capsule is thicker than the posterior surface, it was considered important to know whether the difference in capsule thickness elicited the difference in the rate of depolarization between the anterior and posterior lens fibers after an application of 90 mM K^+, Rb^+, Cs^+ or NH_4^+ test solution.

Selective incubation of either the anterior or posterior surface of isolated lens in Ringer containing 3% collagenase (Sigma, Type I) for 5–20 min at room temperature (Duncan, 1969) resulted in depolarizations of 8–15 mV in both the anterior and posterior potentials. Subsequent application of 90 mM Cs^+ test solution on the enzyme-treated anterior or posterior side showed that the rate of depolarization was still greater in the posterior fibers than in anterior ones and that the time courses of changes of membrane potentials obtained in enzyme-treated lens were also quite same to those in the untreated lenses.

Table 2. Effects of an application of 90 mM K^+, Rb^+, Cs^+ or Nh_4^+ test solution on the membrane potential of lens fibers on the anterior or posterior side of isolated bullfrog lenses exposed to normal Ringer solution (N-Ringer)

	Solution		Experimental (mV)			
Measurement	Anterior side	Posterior side	Control	100 min after		$t_{1/2}$ (min)
Anterior lens fibers	90 mM K	N-Ringer	-93.5 ± 4.4 ($n = 28$)	-10.8 ± 3.5		1.5
	90 mM Rb	N-Ringer	-93.5 ± 3.6 ($n = 25$)	-15.0 ± 3.2	$P < 0.001$	3.1
	90 mM Cs	N-Ringer	-95.0 ± 5.2 ($n = 25$)	-34.8 ± 4.2	$P < 0.001$	18.5*
	90 mM NH_4	N-Ringer	-97.0 ± 4.7 ($n = 24$)	-39.5 ± 4.3	$P < 0.001$	33.0†
Posterior lens fibers	N-Ringer	90 mM K	-94.1 ± 5.4 ($n = 20$)	-10.7 ± 4.8		0.8
	N-Ringer	90 mM Rb	-94.0 ± 3.9 ($n = 18$)	-15.0 ± 4.2	$P < 0.001$	1.4
	N-Ringer	90 mM Cs	-95.6 ± 3.8 ($n = 18$)	-27.5 ± 3.6‡	$P < 0.001$	3.4
	N-Ringer	90 mM NH_4	-96.9 ± 4.3 ($n = 16$)	-30.6 ± 3.4§	$P < 0.001$	4.0

Values listed are the mean ± 1 SD. Numerals inside the parenthesis indicate number of fibers measured in 6–8 lenses. $t_{1/2}$ is the time taken for half depolarization after adding test solutions. * and † were calculated by using the maximal depolarized potential values of the posterior side (‡ and §) after 100 min respectively, since the depolarization of lens fibers at the anterior side is still in progressive phase in both 90 mM Cs^+ and NH_4^+ solutions even at 100 min after the beginning of the perfusion. The significance of the difference of the means between 90 mM K and other test solutions was checked by Student's t-test.

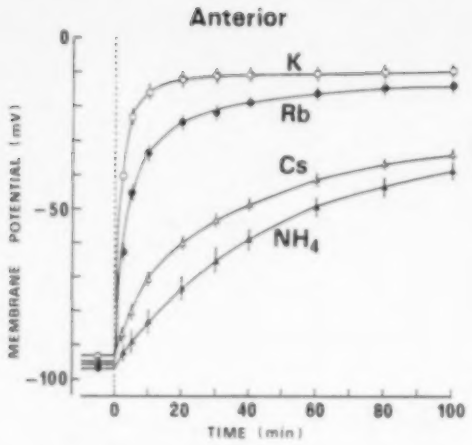

Fig. 3. Effects of 90 mM K^+, Rb^+, Cs^+ or NH_4^+ test solution on the resting membrane potentials of anterior lens fibers. Only anterior side was perfused by these test solutions while the posterior side was perfused by normal Ringer through the experiments. All recordings were obtained from lens fibers at a depth of 200 μm from the anterior surface. Each point represents an average value of between 16 and 25 fibers from 6–8 lenses. Vertical bars for each point show ± 1 SD. Time zero indicates the onset of the test solution. Ordinate: resting membrane potential (mV). Abscissa: time (min).

Changes of the external K^+ concentrations

Taking into account the differences in the time course of K^+ depolarization in anterior and posterior fibers shown in Figs. 3 and 4, the potential measurements were made on both sides after soaking in the various K^+ test solutions more than 100 min. The measured membrane potentials were plotted against log $[K]_0$ (extracellular K^+ concentration) (Fig. 5). It is evident from this figure that the anterior and posterior fibers were depolarized to the same steady-state potentials with respect to the different $[K]_0$. The slope of the relationship for both fiber types was approximately 58 mV for a 10-fold change in $[K]_0$ between 10 and 120 mM. This agrees well with the diffusional potentials for K^+ predicted by the Nernst formula (dashed line of Fig. 5)

$$E_K = \frac{RT}{zF} \ln \frac{[K]_0}{[K]_i} \qquad (1)$$

where F, R and T have their usual significance and $[K]_i$ is the intracellular K^+ concentration. At $[K]_0$ less than 10 mM, however, the lens fibers depolarized and the slope relating the membrane potentials to $[K]_0$ became smaller than that predicted from the Nernst equation. Lens fibers were depolarized to zero potential at 135 mM $[K]_0$ yielding an estimate of 135 mM for $[K]_i$ based on the Nernst formula. Similarly if $[K]_i$ is 135 mM, $[Cl]_i$ can be estimated at 2.22 mM from the Donnan equation. Therefore, it is interesting to know whether the behaviour of the membrane potential in lens fibers is described by a constant-field equation (Goldman, 1943). If $P_K[K]_i$ is much greater than $P_{Na}[Na]_i$ may be neglected (Hodgkin and Horowics, 1959), the following simplified equation can be derived from the constant-field theory,

$$V_m = \frac{RT}{zF} \ln \frac{[K]_0 + (P_{Na}/P_K)[Na]_0}{[K]_i} \qquad (2)$$

where V_m is the membrane potential, P_K and P_{Na} are permeability constants for K^+ and Na^+, and P_{Na}/P_K is the relative permeability ratio for Na^+ to that for K^+. The curve in Fig. 5 shows the theoretical relation, calculated from equation (2) by choosing the values 135 mM for $[K]_i$ and 0.01 for P_{Na}/P_K respectively, which indicates that all of the experimental points at 20°C lie on a theoretical curve.

DISCUSSION

There are considerable differences in anatomical barriers to ion diffusion pathway from either anterior or posterior sides to the lens interior. From the anterior surface, ions must diffuse across the capsule layer, an epithelial cell layer and outer lens fiber layers. Conversely, on the posterior side, ions diffuse across both the capsule and the lens fiber layers encountering no epithelial barrier on this side. In vertebrate lens, the depolarization of lens fibers are elicited by the diffusion of ions from the bulk solution to the lens surface membrane, since almost 50% of the lens fiber cell surface consists of junctional complexes and the lens fibers are electrically coupled to one another (Duncan, 1969; Philipson *et al.*, 1975; Kuszak *et al.*, 1978; Jacob and Duncan, 1981). In the present experiments, the depolarization produced by high K^+, Rb^+, Cs^+ or NH_4^+ test solution was faster in posterior fibers than in anterior fibers in the normal lens and in the decapsulated lens preincubated in collagenase. This result suggests that the lens capsule layer provides little or no barrier for ionic diffusion from either anterior or posterior surface. In fact, lens capsule is highly permeable to ions (Takeguchi and Nakagaki, 1969, 1970).

As described above, the posterior lens fibers depolarized at a faster rate than in anterior ones in high K^+, Rb^+, Cs^+ and NH_4^+ solutions. Given enough immersion time, generally 100 min in the test solution, however, there was no difference in the "steady state"

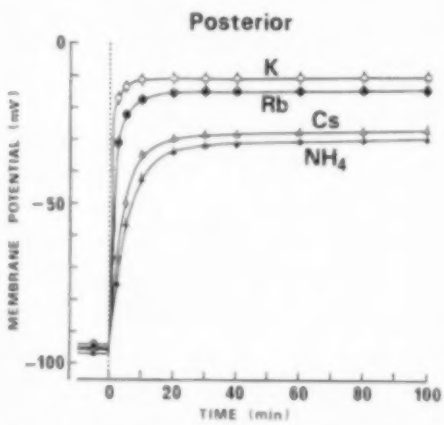

Fig. 4. Effects of 90 mM K^+, Rb^+, Cs^+ or NH_4^+ on the resting membrane potentials of posterior lens fibers. The test solutions were applied to the posterior side only. Each point represents the mean of between 16 and 28 lens fibers from 6–8 lenses. Vertical bars indicate ± 1 SD of the mean. Time zero indicates the onset of the test solutions.

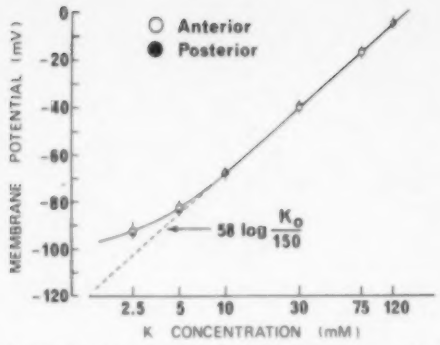

Fig. 5. Relationship between the resting membrane potentials of anterior (○) and posterior (●) lens fibers and log $[K]_o$. Each point is the mean of measurements for between 40 and 76 fibers from 8–14 lenses. Vertical bars for each point represent ±1 SD of the mean. The dashed line represents the predicted relationship based on the Nernst equation yielding a line of slope of 58 mV per decade change in ion concentration at 20°C. Ordinate: resting membrane potential (mV). Abscissa: external K^+ concentration (mM).

membrane potentials of fibers of the both sides (Figs 3–5). We believe, therefore, that the considerable delay of the depolarization in the anterior fibers as compared with the posterior fibers in high cation test solutions results from the diffusional restriction for ions of the anterior epithelial cell layer.

When the anterior surface of frog lenses is electrically isolated from the posterior surface in a divided chamber (Candia *et al.*, 1970; Kinsey and McGrady, 1971; Delamere and Duncan, 1979) or by immersing in liquid paraffin (Duncan *et al.*, 1977), an asymmetrical potential of 8–10 mV (anterior face positive), which is attributed to the anterior "electrogenic" pump, was observed. However, in the present experiments, there were no asymmetry potentials between the anterior and posterior fibers. Similar results were reported in zonule-suspended frog lenses (Brindley, 1956; Duncan *et al.*, 1977). These results suggest that the lens constitutes an isopotential system since a short circuit occurs through the region of the suspensory ligaments in the zonule-suspended preparation and through the lens capsule (Takeguchi and Nakagaki, 1970) and deficient seal between the lens and the chamber in the isolated preparation.

The relative permeability ratio, P_{Pa}/P_K, reported by Duncan (1969) and Delamere and Duncan (1977) for the frog lenses (*Rana temporaria* and *Bufo bufo*) was about 0.2. The bovine lens showed very large P_{Na}/P_K values such as 0.5–0.6 (Duncan *et al.*, 1977). Thus, they explained the low membrane potential in bovine lens resulting in a high Na^+ permeability. In the present experiments, however, the measured lens fiber potentials agreed very well with the E_K at more than 10 mM $[K]_0$ and the P_{Na}/P_K value was considerably small (0.01).

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A POSSIBLE ROLE FOR THE THYROID IN REPRODUCTIVE INHIBITION IN LABORATORY POPULATIONS OF THE PRAIRIE DEERMOUSE (*PEROMYSCUS MANICULATUS*)

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Abstract—1. Serum thyroxine concentrations were determined and found to be reduced in reproductively inhibited deermice selected from controlled growth laboratory populations.

2. Daily injections of 1 μg thyroxine in reproductively inhibited male deermice were shown to significantly increase testis weight and the number of spermatids with acrosome formation.

3. Daily feeding of thyroxine to a controlled growth population promoted an increase in the mean weights of the reproductive organs of both sexes that was intermediate between reproductively inhibited and capable deermice. Also, two females in the population produced the first surviving young after 250 days of inhibition.

INTRODUCTION

The hypothesis that a hyperfunctional pituitary–adrenal axis is the primary cause of inhibited reproductive function in dense populations of some animal species is widely accepted. However, our investigations using laboratory populations of the prairie deermouse (*Peromyscus maniculatus bairdi*) have consistently demonstrated that reproductive inhibition in this species is not associated with hypertrophied adrenals. Also, the physical condition of inhibited animals resembles an immature state in terms of reproductive organ and general body development. Earlier evaluation of serum concentrations of LH, FSH, testosterone, ACTH and corticosterone have led us to investigate the possibility that the inhibited state is promoted and maintained by more than these factors. This report is of a series of experiments that were designed to define the extent to which the thyroid gland and its secretions may control reproductive inhibition.

MATERIALS AND METHODS

Animal maintenance

The animals used in this study were prairie deermice obtained from a laboratory colony in which no sibling matings were allowed. Colony animals were kept in 12.5 × 27 × 14.5 cm plastic, wire-top cages and were illuminated daily by bright light (four 40 W fluorescent tubes) for 14 hr (0700–2100 hr) and in darkness for 10 hr. Control animals were placed on the same light cycle as the experimental populations after they were paired. This cycle was the same duration of light and dark, but the period of light was from 2200 hr to 1200 hr. This permitted sampling during the period around the light to dark transition which is at the onset of the dark-active period for this species. Control animals were paired in "no-contact" wire cages (12 × 26 × 14 cm) which permitted the exchange of olfactory, visual and auditory cues, but prevented all tactile exchange through a double thickness (2 cm) of hardware cloth. Previous experience with this cage system has shown that females cycle normally and that pregnancy is eliminated as a complicating factor (Bradley and Terman, 1979). All control and population animals were maintained on food (Wayne Laboratory Animal Diet) and tap water in excess of utilization. Yearly animal room temperature fluctuations were maintained between 21 and 30°C, and daily fluctuations were rarely more than 2°C.

Experimental populations were founded by four pairs of reproductively proven mice, with all animals taken from different litters. The first litter of those females which were pregnant upon introduction into the enclosures were discarded so that only young produced within the population context would remain as part of that population. Population enclosures consisted of a corrugated aluminium wall within a circular stainless steel base 1.5 m in dia. The floor area was covered with wood shavings and eight plastic nest boxes were provided in each enclosure (Terman, 1969).

Collection of tissues

Because decapitation blood yields are too low, blood samples were taken from control and population animals in Experiment I by first rapidly anesthetizing each with diethyl ether and then collecting blood from the dorsal aorta in the region of the renal artery. All animals were sacrificed within one-half hour after initial disturbance. Döhler *et al.* (1977) reported that serum levels of triodithyronine in rats do not fluctuate significantly until 60 min after disturbance and that thyroxine concentration never fluctuated significantly. Sera, which were obtained by centrifugation of blood samples for three minutes at 9000 *g*, were stored at below −30°C until assayed for thyroxine concentration.

In all animals, the testes, seminal vesicles, or ovaries and uteri were fixed in 10% buffered formalin for at least 72 hr and were cleaned of surrounding tissue before being weighed or examined. The formalin-fixed weights of the reproductive organs were measured on a Cahn electrobalance interfaced with a Wang 700B programmable calculator.

Radioimmunoassay methods

Total serum thyroxine concentrations were determined by a competitive binding technique using the T4 RIA (PEG) Diagnostic Kit (Abbott Laboratories). This method utilized iodine-125 labeled thyroxine, thyroxine antisera and 8-anilino-1-napthalenesulfonic acid to extract bound thyroxine and to prevent rebinding. The antisera–thyroxine complexes were precipitated and separated with polyethylene glycol and centrifugation at 1000 *g*. Supernates in each assay tube were decanted and the net radioactivity of iodine-125 thyroxine in

the complexes were counted on a Biogamma II (Beckman Instruments) to a 2% error. Serial dilutions of *Peromyscus* sera gave a linear plot that was parallel to, and not significantly different from, the curve produced with standard reagents supplied with the kit.

Histology

The formalin fixed testes from Experiment II were embedded in paraplast (Scientific Products). One testis was randomly selected from each male and sectioned at 7 μm for a distance of 250 μm through the largest diameter. All sections were stained with eosin and hematoxylin and mounted in Permount (Fisher). The sections were evaluated at 40 × using a Zeiss Photomicroscope II.

The major and minor axes of the testes cross-sections were determined with an optical micrometer to find the largest section. A transect line was established along the major axis of the largest section using the center line of the optical micrometer, as described by Bradley and Terman (1981a). The contents of all seminiferous tubules touching the transect were evaluated. In the subsequent statistical analysis of the seminiferous tubules, only those tubules with a minor to major axis greater than 0.85 were used in order to reduce the error due to tangential sections. The seminiferous tubules on the transect were examined at 400 × to determine the number of spermatids with any acrosome formation. The total number of cells (exclusive of Sertoli cells) along the transect were also counted.

Statistical methods

Comparisons of serum thyroxine concentrations, body and reproductive organ weights in control and population animals were made using the Student's *t*-test, or a Mann–Whitney U test, if an *F*-test showed significant heterogeneity of variance. All values are expressed as means $\pm$ the standard error of the mean. Analyses were run to determine if correlations existed between the thyroxine concentrations and age, reproductive status and the reproductive organ and body weights of population mice. A probablility of less than 0.05 was considered significant in all cases.

Experimental protocol

Experiment I was designed to establish the degree of difference in serum thyroxine concentrations, and reproductive organ and body weights, between reproductively inhibited animals from populations and controls maintained as pairs. Population animals were sacrificed at 123 ± 3 days of age. Control animals were paired at 82 ± 2 days of age, and then placed into "no-contact" cages for 30 ± 2 days and subsequently sacrificed at 120 ± 2 days of age. At the time of population sampling, a 40 W long-wave u.v. lamp was used to identify mice which had been marked at least 24 hr earlier with a non-toxic u.v.-sensitive dye (Raytech Industries). All population and control animals were sacrificed between 1000 and 1100 hr and sera were treated as described above. The experiment used 18 pairs of controls and 22 pairs of population mice.

Experiment II was designed to measure the responses of reproductively inhibited population male deermice to an injected dose of thyroxine. Experimental animals were taken from the largest population used in Experiment I. Each animal was paired with another population male of the same age for the purpose of receiving treatments. One animal of each pair was given daily injections (i.p.) of 1 μg l-thyroxine (Sigma) in 0.1 ml 0.9% saline (pH 8.0) for 21 days. The control from each pair was given daily sham injections with the saline vehicle for the same time period. All injections were given at the start of the bright period (2200 hr) and were complete for each animal within three minutes after the identification of the marked animal with u.v. light. The treatment animals were maintained within the population for the duration of the experiment. A total of 12 animals were treated in this experiment.

In Experiment III another experimental population was founded by the method previously described and was used to study the effects of the administration of thyroxine on the reproductive condition of the entire population. This population had been founded in July 1981 and was 320 days old at the outset of this study. At the beginning of the treatment period, the population consisted of four male and two female founders, and five males and eight females born into the population during its growth phase. No increase in population size had occurred during the previous 236 days. At the outset of the experiment, all food was removed from the enclosure and then a weighed amount was returned. After seven days, all food was again removed, weighed and the amount of food consumed per animal was calculated. In a like manner, water consumption was also measured. At the outset of the experiment, and every seven days thereafter, each animal was removed from the population, weighed and checked for testis position or vaginal perforation and evidence of pregnancy.

Based upon the initial average daily water consumption value, a stock solution of l-thyroxine (Sigma) was made up in distilled water (with 4 N NaOH to obtain pH 8.0) to provide an average daily dose of 1 μg of thyroxine per animal. Weekly monitoring of water intake provided data to modify the concentration of drinking fluid to maintain this dose rate. All food and the thyroxine containing water were measured at weekly intervals at the same time as the population check for reproductive condition.

The experimental period involved a seven day pretreatment to estimate normal intake, and a 28 day period of daily thyroxine administration. At the end of the treatment period, all animals in the population were sacrificed and their reproductive condition was evaluated from the formalin-fixed weights of their reproductive organs.

RESULTS

Experiment I

In this experiment it was found that population females tended ($P < 0.1$) to have a lower mean serum thyroxine concentration in comparison with control females (Table 1), and that the mean thyroxine concentration for population males was significantly ($P < 0.03$) lower relative to control males (Table 2). The mean reproductive organ weights (testes, seminal vesicles, ovaries and uteri) in population animals were all highly significantly ($P < 0.01$) smaller than the respective values for reproductively capable controls. The mean body weights of both males and females from populations were significantly ($P < 0.01$ and $P < 0.01$, respectively) smaller than the corresponding control animals (cf. Tables 1 and 2).

Experiment II

Population males given daily injections of 1 μg of thyroxine for 21 days did not have significantly different body and seminal vesicle weights compared with sham injected controls. However, thyroxine injected males did have significantly ($P < 0.05$) larger absolute and relative mean testes weights than sham treated males, and highly significantly ($P < 0.001$) larger absolute and relative adrenal weights (Table 3).

Histological evaluation of the testes from thyroxine injected males indicated dramatic development when compared with sham injected males (Table 4). The mean width of the largest testes cross-section was significantly ($P < 0.01$) larger than the control value. The mean number of seminiferous tubules on the transect line was also significantly ($P < 0.01$) greater in

thyroxine injected males than in sham injected males. The mean number of all cells on the transect of thyroxine injected animals was significantly ($P < 0.001$) larger than in shams and the range of values (16.0–21.3 vs 7.7–13.4) did not overlap. The mean number of spermatids in all stages of acrosome development was significantly ($P < 0.001$) higher in thyroxine injected males compared with shams. There was no overlap in the range of these values (12.1–16.1 vs 2.7–8.0).

Experiment III

The mean daily rate of food consumption tended ($P < 0.1$) to increase (2.35 g/animal per day, pretreatment vs 3.33 g/animal per day in the last week), and the water consumption remained not significantly different during the period of thyroxine administration. The mean dose of thyroxine over the 28 day treatment period was 0.99 ± 0.05 μg/animal per day. The mean body weight of both sexes remained not significantly different during the pretreatment and first three weeks

Table 1. Serum thyroxine concentration, body weight, and ovary and uterus weights in control and population females (values are mean ± SEM)

Treatments	Serum thyroxine (μg/100 ml)	Body weight (g)	Ovary weight (mg)	Uterus weight (mg)
Control females ($n = 18$)	2.5 ± 0.16	16.2 ± 0.48	17.8 ± 1.41	47.8 ± 4.61
Population females ($n = 22$)	2.1 ± 0.12*	12.4 ± 0.35†	5.3 ± 0.52†	9.8 ± 2.25†

*$P < 0.01$.
†$P < 0.001$.

Table 2. Serum thyroxine concentration, body weight, and testes and seminal vesicle weights in control and population males (values are mean ± SEM)

Treatments	Serum thyroxine (μg/100 ml)	Body weight (g)	Testes weight (mg)	Seminal vesicles weight (mg)
Control males ($n = 18$)	3.1 ± 0.24	17.1 ± 0.40	299.3 ± 20.28	145.5 ± 14.76
Population males ($n = 22$)	2.4 ± 0.12*	14.7 ± 0.63†	92.8 ± 13.50‡	12.8 ± 4.46‡

*$P < 0.03$.
†$P < 0.01$.
‡$P < 0.001$.

Table 3. Effects of thyroxine on body weight, and absolute and relative testes, seminal vesicles, and adrenals weights (mean ± SEM)

			Testes		Seminal vesicles		Adrenals	
Treatment	Age (days)	Body weight (g)	Abs (mg)	Rel (mg/g)	Abs (mg)	Rel (mg/g)	Abs (mg)	Rel (mg/g)
Sham injected (6)	287.7 ± 33.60	16.2 ± 0.87	171.8 ± 34.15	10.4 ± 1.87	144.8 ± 46.90	8.7 ± 2.79	2.2 ± 0.07	0.1 ± 0.01
Thyroxine injected (6)	287.7 ± 33.60 n.s.	17.6 ± 0.91 n.s.	277.8 ± 25.63*	15.9 ± 1.58*	170.7 ± 17.33 n.s.	9.8 ± 1.10 n.s.	4.1 ± 0.05†	0.2 ± 0.00†

*$P < 0.05$.
†$P < 0.001$.
n.s., not significant.

Table 4. Effects of thyroxine on testes cross section width, mean number of seminiferous tubules with a/b ratio of >0.85, total number of cell on transect, and number of spermatids with acrosomes on transect (mean ± SEM)

Treatment	Width of testes cross section (μ)	Mean number of seminiferous tubules with a/b ratio of > 0.85	Total number of cells on transect	Number of spermatids with acrosomes on transect
Sham injected (6)	1666.5 ± 136.26	6.5 ± 0.42	10.5 ± 0.92	5.6 ± 0.94
Thyroxine injected (6)	2260.5 ± 121.02*	8.5 ± 0.34*	18.6 ± 0.71†	13.6 ± 0.56†

*$P < 0.01$.
†$P < 0.001$.

Table 5. The mean adrenal weight, and testis and seminal vesicle weight, or ovary and uterus weights (mean ± SEM) of Experiment III population animals fed 1 μg thyroxine daily for 28 days

	Adrenal (mg)	Testis (mg)	Seminal vesicles (mg)	Ovary (mg)	Uterus (mg)
Males (9)	3.7 ± 1.95	141.5 ± 39.40	53.6 ± 19.78	—	—
Females (8)*	2.3 ± 0.06	—	—	6.3 ± 0.66	15.3 ± 7.53

*Exclusive of two recently pregnant animals.

of the treatment period, but the mean weight fell significantly ($P < 0.001$) in the fourth week in both sexes. Table 5 presents the various organ weights obtained after 28 treatment days.

On treatment day seven, a founding female (No. 403) delivered five live young, four of which survived until the end of the treatment period. On treatment day nine, a founding female (No. 401) delivered three live young, all of which survived to the end of the treatment period. The first of these young represented the first surviving litter born into the population for the previous 250 days.

DISCUSSION

The drastic degree of reproductive inhibition observed in the animals that were selected from populations and used in these experiments was similar in all respects to that reported in other studies from our laboratory (Terman, 1965, 1973, 1979; Bradley and Terman, 1979, 1981 a, b, c). In some of these earlier studies, it was shown that at least 90% of the young deermice born into populations fail to mature reproductively, both during the growth phase of the population and when numerical growth has ceased. These present data confirm once again that there is a highly significant reduction in reproductive capability in both sexes selected from laboratory populations. Our earlier studies have shown that inhibited females have an ovarian histology, and serum FSH and LH concentrations that are characteristic of prepubertal animals that do not have LH surges and do not ovulate (Bradley and Terman, 1981b). Similarly, the serum FSH, LH and testosterone concentrations and testis histology in population inhibited males indicates little, or no, spermatogenesis, lowered serum LH and testosterone, and elevated serum FSH concentrations (Bradley and Terman, 1981a, b). This constellation of observations, including reduced body and gonad weight, all are typical of normally maturing 40 day old pre-, or peri-pubertal males.

We felt that the observed changes could be most simply explained if the population animals were thyroid deficient. Experiment I was designed to test whether the serum thyroxine concentration might reflect a reduction in thyroid activity. The results of this experiment show that there is, indeed, a significant ($P < 0.03$) reduction in the mean serum thyroxine concentration in population males, during at least one time of day. Similarly, the mean serum thyroxine concentration in inhibited females tended ($P < 0.1$) to be lower than their control value. Evaluation of serum thyroxine at only one time of day cannot establish hypothyroidism, nor does it suggest an underlying cause for the condition. Therefore, investigations are currently underway to evaluate the serum concentrations of thyroxine (T4), triiodothyronine (T3) and TSH in the serum of population inhibited animals at several selected times of the day, and to compare these values with corresponding values from reproductively capable controls.

Evidence for the precise way that environmental stress factors might depress or disrupt thyroid function is equivocal. For example, there are contradictory reports on the response of the thyroid to acute stress. Döhler *et al.* (1977) reported that serum T4 fluctuated insignificantly and T3 was elevated along with serum corticosterone after disturbance stress in rats. But Chan *et al.* (1978) reported a sharp, early rise in T4 after surgical stress in rats. Langer and Lichardus (1969) found that TSH secretion and thyroid function were decreasing simultaneously with increased ACTH secretion. But Fenske and Wutke (1977) found no evidence of any stress influence in rats.

More relevant to the population context studied here, are the effects of chronic stress. Early work suggested that a chronic immobilization stress for 24–72 hr led to an inhibition of thyroidal I-131 release (Brown-Grant *et al.*, 1954). This inhibition was shown to be released after pituitary stalk section, which was presumed to disrupt the hypothalamic control (Brown-Grant *et al.*, 1957). Mason *et al.* (1961) found that chronic emotional stress increased plasma protein bound iodine in monkeys, whereas, Volpé *et al.* (1961) reported that emotional tension and anxiety in humans failed to produce any significant fluctuation in serum protein-bound iodine.

It has been known for some time that there is a significant inter-relation between thyroid and adrenal function via pituitary control. Retiene *et al.* (1968) and Otsuki *et al.* (1973), have demonstrated a reciprocal relationship between ACTH and TSH secretion in rats. ACTH and hydrocortisone doses were found to inhibit the release of TSH and thyroid hormone in man (Levin and Daughaday, 1955; Nicoloff *et al.*, 1970), and exogenous dexamethasone reduced plasma TSH concentration in rats (Wilber and Utiger, 1969). Collectively, these and many similar reports suggest that stress may be capable of indirectly inhibiting thyroid activity via an increase in pituitary–adrenal activity.

A recent report of adrenal activity in reproductively inhibited deermice (Bradley and Terman, 1981c) demonstrated a significant increase in the serum concentration of corticosterone, but there was no significant increase in adrenal hypertrophy or hyperplasia suggestive of elevated ACTH concentrations. In addition, there is now preliminary information that 1 hr prior to the light to dark transition (which is the onset of activity for this species) ACTH concentrations in reproductively inhibited animals are not significantly different from reproductively capable controls. Also, electron microscopic evaluation of the adrenals of

ACTH treated deermice show a markedly different appearance compared with adrenals from reproductively inhibited or reproductively capable deermice (Coppes, 1980). Thus, there may be tentative reasons to suspect that thyroid function is not depressed due to elevated serum ACTH. It should be mentioned that serum thyroxine concentrations can be reduced by many environmental factors, including temperature. Also, hypothyroidism can promote elevated serum glucocorticoids via decreasing catabolism and excretion (Brown *et al.*, 1958; Peterson, 1958; Miller *et al.*, 1970). We are currently investigating whether any environmental factors may be directly affecting thyroid function. However, we continue to be aware that ACTH may be elevated at other times of the day, or that it may be chronically elevated compared with a normal circadian pattern. Further investigations are clearly indicated.

It has been long established that the thyroid is involved in maintaining normal reproductive function, and that impairment of thyroid activity may be inhibitory to reproduction (Maqsood, 1950; Hoar *et al.*, 1957; Parrott *et al.*, 1960). More recently it has been reported that a reduction in the secretion and plasma levels of gonadotropins has been associated with hypothyroidism (Hagino, 1971; Larochelle and Freeman, 1974; Buchanan *et al.*, 1977). Likewise, Dunn *et al.* (1976) have shown that the daily rhythmic release of LH and prolactin in rats was altered by thyroidectomy.

Because of the strong likelihood that the thyroid function is depressed in reproductively inhibited population deermice, we were interested in determining if thyroxine replacement therapy, given alone, could promote any recovery of reproductive function. In Experiment II, we controlled for the wide range of ages that were present within the population by pairing the male deermice by age to receive either the thyroxine or the sham injections. All of the animals selected were found to have non-scrotal testes at the outset of the experiment.

The effect of the injected daily dose of 1 μg thyroxine on the testes was marked. The absolute and relative testis weights were significantly ($P < 0.05$) larger in the thyroxine treated animals compared with sham injected animals of the same age. This increase in weight was reflected in the increase in the width and the numbers of cells in the seminiferous tubule cross-section. Of particular interest was the observation that the thyroxine treated animals had significantly ($P < 0.001$) greater number of spermatids with acrosomes than the inhibited animals. Comparing these present data with earlier work from our laboratory on the histological condition of the inhibited deermouse testis, strongly suggests that the thyroxine treatment restored a significant degree of spermatogenesis. The fact that the testosterone sensitive seminiferous tubules (Bradley and Terman, 1981a) were not significantly larger may indicate that testosterone concentrations were not fully restored, and that the thyroxine treatment only caused a partial restoration of testis function. It is also possible that the duration of the thyroxine treatment was not sufficient to cause the sequential restoration of the Leydig cell function, testosterone secretion and, finally, seminal vesicle development.

The purpose of Experiment III was to see if the partial restoration of testis function seen in Experiment II would be translated into the recovery of reproductive function for an entire thyroxine treated population. A population that had controlled growth for 243 days was selected, and thyroxine was administered in the drinking water at a mean dose rate comparable to the injected thyroxine dose in Experiment II. Shortly after the onset of the thyroxine treatment two of the original founding females became pregnant. The timing of those pregnancies was such that insemination occurred in the prethyroxine treatment period, and so in these cases, induction of the ovulation and spermatogenesis were not due to the thyroxine treatment. However, each of these females had been recorded as being pregnant a few times during the 234-day period when the population was no longer growing, and in every case their young were never found. Therefore, at least in these females, the role of thyroxine was not to promote ovulation or mating, but to facilitate the maintenance of the pregnancy and/or to promote adequate lactation and maternal care. The experiment was terminated after 28 days of thyroxine treatment in order to determine the reproductive condition of the entire population.

A comparison of the various body and organ weights for all of the thyroxine treated adult animals in the population (21 animals, aged 380–261 days) with similar data recently published by Bradley and Terman (1981b) from 120-day-old males and females taken either from populations, or from reproductively capable control pairs, reveals some striking details. The mean adrenal weights of all thyroxine treated males were heavier than both the control and the population inhibited animals from the earlier study (3.7 ± 1.95, 3.0 ± 0.13, and 2.1 ± 0.10 g, respectively). However, the mean adrenal weight for females in this study was not significantly different when compared with the control and population animals from the other study (2.3 ± 0.06, 2.7 ± 0.12, and 2.4 ± 0.11, respectively). We believe that the adrenal response observed in both Experiments II and III are the typical pharmacologic responses to exogenous thyroxine that have been previously described (Deane and Greep, 1947; Steinmetz and Beach, 1962).

The testes weight and seminal vesicle weights of thyroxine treated animals were intermediate between values for reproductively capable controls and reproductively inhibited animals from populations in the earlier study (testes: 141.5 ± 39.4, 310.3 ± 12.3 and 44.9 ± 7.1 g; seminal vesicles: 53.5 ± 19.8, 150.3 ± 7.7 and 2.6 ± 0.7 g, respectively). Likewise, the ovary and uterine weights of the thyroxine treated females (exclusive of the recently pregnant females Nos. 401 and 403) were intermediate between the reproductively capable controls and the reproductively inhibited animals taken from populations in the other study (ovary: 6.3 ± 0.66, 16.2 ± 1.00 and 3.7 ± 037 g; uterus: 15.3 ± 7.5, 46.8 ± 5.8, and 7.0 ± 2.0 g, respectively).

The range of individual values for the reproductive organ weights also supports the notion that these organs were restored in the direction of the reproductively capable animals in the Bradley and Terman (1981b) study. All of the seminal vesicle weights in the thyroxine treated males were above the mean value obtained in the Bradley and Terman (1981b) study for

inhibited males selected from populations. Likewise, all of the ovary weights were above the mean value determined for inhibited females in that earlier study. No statistical comparisons were made between the data from this study and the previous study, because of the confounding variables of different age ranges and the initial experimental design objectives for the two studies. However, given those limitations, differences are clearly apparent and reveal a shift toward enhanced gonad activity in thyroxine treated population animals. Whether this recovery would have been complete if the dose level had been of longer duration is not known.

The results of these experiments strongly suggest that reproductively inhibited animals from populations have reduced thyroid activity. The possibility that thyroid function may be central in promoting and/or maintaining reproductive inhibition, and hence population growth, is a relatively unexplored possibility. our demonstration that thyroxine given alone to inhibited animals within populations promoted a partial recovery of some of the depressed systems, must be differentiated from a pharmacologic response that is unrelated to the mechanism of population induced inhibition. Projects now underway will more clearly define the question of primary vs secondary hypothyroidism and thus be suggestive of whether an environmental factor present within the population context may be directly influencing thyroid function.

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SEASONAL RELATIONSHIPS OF THYROID, SEXUAL AND ADRENOCORTICAL HORMONES TO NUTRITIONAL PARAMETERS AND CLIMATIC FACTORS IN WHITE-TAILED DEER (*ODOCOILEUS VIRGINIANUS*) OF SOUTH TEXAS

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Abstract—1. Annual cycles of serum testosterone (T), estradiol 17-β (E), thyroxine (T_4), triiodothyronine (T_3) and cortisol (C) and their relationship with dry matter intake (DMI), ambient temperature and daylength (DL) were examined in four male and four female white-tailed deer.

2. Serum T in bucks correlated ($P < 0.05$) with DMI during the rut. In does, E correlated ($P < 0.05$) with DMI and body wt. Both serum T and E were DL and temperature dependent.

3. Serum T_4 in bucks correlated ($P < 0.05$) with body wt and increasing temperature while T_3 correlated ($P < 0.05$) with DMI and body wt. In does, T_4 was significantly ($P < 0.05$) correlated with DMI and body wt, while T_3 was not.

4. Serum C levels were not correlated ($P > 0.05$) with either DMI or body wt.

5. It appears that serum T_4 and T_3 in bucks and T_4 in does offer the best year-round indicators of nutritional stress in deer.

6. Male DMI was temperature and DL dependent. In does, only a short-term effect on DMI was found.

7. After the breeding season in bucks, and throughout the year in does, DL and temperature may control intake via the thyroid hormones.

INTRODUCTION

White-tailed deer (*Odocoileus virginianus*) in North America display a seasonal fluctuation in feed consumption and body wt (French *et al.*, 1960; Holter *et al.*, 1977; Moen, 1978). Both male and female deer voluntarily decrease their intake in the winter. It is believed that this hypophagia assists white-tails in reproduction and environmental adaptation (McMillin *et al.*, 1980). In boreal regions, feed consumption generally increases in the summer and is suppressed in adult males during the fall rut or breeding season. There is a short compensatory intake period after the rut, followed by restricted intake the remainder of the winter. In southern environments, there is no winter compensatory period, but there is an additional mid-summer intake suppression, probably due to high temperature and humidity (Short *et al.*, 1969; Wheaton and Brown, 1983). In addition to these geographical differences in feed intake, the latitude also affects the timing of the breeding season (Robinson *et al.*, 1963).

In south Texas, because of its more temperate climate, the deer have a more complex environment to which to adapt (i.e. mild winter cold stress but severe summer heat stress). Meyer (1982) reported that summer was more critical to these deer than winter in terms of the nutritional adequacy of the diet. Marked reductions in feed consumption by sheep and cattle can be caused by high environmental temperatures or rations with a high calorigenic effect. Thus, thermostatic mechanisms may dominate regulation of feed intake independently of other factors (Conrad, 1966).

The annual cycles of testosterone (T), estradiol 17-β (E), thyroxine (T_4) triiodothyronine (T_3), and cortisol (C) have been reported in deer (McMillin *et al.*, 1974; Bubenik *et al.*, 1975a; Bubenik *et al.*, 1977; Mirarchi *et al.*, 1981). Testosterone levels in bucks are highly correlated with the rut (McMillin *et al.*, 1974; Bubenik *et al.*, 1977; Mirarchi *et al.*, 1977) while E is the principal ovarian steroid regulating mating activity in does (Wade, 1972). In deer, T_4 and T_3 are metabolic hormones correlated with feed intake (Seal *et al.*, 1978). They also have been used as indicators of nutritional stress (Bahanak *et al.*, 1981). Cortisol is a main metabolic steroid in ruminants regulating protein and carbohydrate metabolism and also has been used as an indicator of general stress in deer (Bubenik *et al.*, 1977).

While McMillin *et al.* (1980) reported some hormonal relationships with hypophagia in northern white-tailed deer, no one has studied this phenomenon in southern white-tails. This project was designed to investigate the annual cycles of hormonal changes and their interrelationship with hypophagia and ambient climate in south Texas white-tailed deer.

MATERIALS AND METHODS

Four male and four female 1½-year-old white-tailed deer were housed individually in 5 × 5 m covered pens and fed a complete pelleted diet† (18.93% C.P. and 2.45 Kcal DE/g DM) *ad libitum* from 1 October 1979 to 30 September 1980. Average daily dry matter intake (DMI) was recorded weekly

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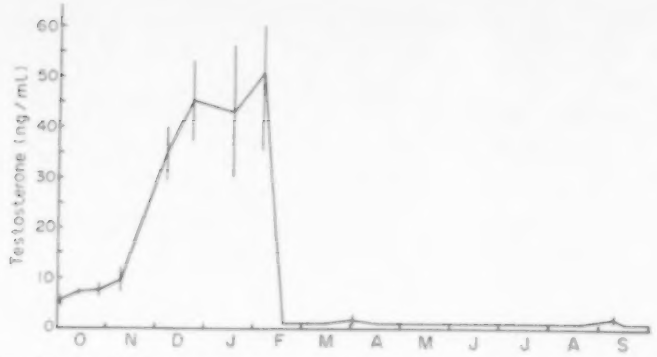

Fig. 1. Annual cycle of serum testosterone, mean (± SD) of four male yearling white-tailed deer.

while body wt was measured and blood samples were collected bi-weekly. For blood collection, the deer were tranquilized with xylazine hydrochloride (Rompun), and samples were taken from the jugular vein by venepuncture. After centrifugation, serum samples were stored at −20°C for further analysis.

Temperature data were taken from the climatological substation at Texas A & I University, Kingsville. Daylength (DL), rainfall and relative humidity (RH) data were provided by the U.S. Naval Air Station in Kingsville.

Testosterone, E, T_4 and T_3 were measured on duplicate samples using radioimmunoassays kits from Wien Labs, Inc.,* while C was measured by an assay from Corning Medical Scientific Co.† The T and E assays utilized anti-hormone–protein complex antibodies and ^3H as a tracer. The T_4 and T_3 assays utilized anti-T_4 or T_3–protein complex antibodies and ^{125}I as a tracer. The C assay utilized anti-cortisol–protein complex antibodies and ^{125}I as a tracer.

Due to the sample size, bi-weekly hormonal levels and weekly intake data were pooled into six periods of 2-month duration and statistically analyzed by analysis of variance with a SPSS computer program. Pearson correlation coefficients were analyzed by deer and by sex to determine correlations between hormones, intake and climate data. The statistical significance level was set at $P < 0.05$.

Three climatological periods in the study were: Period 1, from October through January, as temperature and DL decreased; Period 2, from February through May, as temperature and DL increased; and Period 3, from June through September, as temperatures were generally greater than 27°C and DL decreased. In determining these periods, biological events were also considered, since the first period includes the rut or breeding season, the second period is the period of sexual quiescence, and the third period corresponds to the antler growth period in bucks. Since attempting to correlate blood values with contemporary environmental data might encounter substantial variance due to prior events (Seal *et al.*, 1972), lagged climatic data were also correlated with bi-weekly blood parameters in addition to divided periods. Temperature and DL from the previous 1–7 weeks was lagged to examine short-term (1–2 week) and long-term (7 week) effects on hormonal changes and DMI (i.e. T1 was average temperature data from current week, T2 was averaged temperature data from current and previous week, etc.).

RESULTS

Annual cycles in serum hormone levels

Serum T in bucks (Fig. 1) increased slightly from mid to late autumn, then increased dramatically and peaked (50.6 ng/ml) during late winter. In February, it dropped abruptly to a baseline (1.0 ng/ml) until autumn. The changes of T levels confirm the study of testicular changes in Texas by Robinson *et al.* (1963) in which changes lagged 2 months behind those of Northern white-tails (Mirarchi *et al.*, 1977; McMillin *et al.*, 1980).

Serum E levels in does (Fig. 2) increased gradually from October until early winter, then dropped abruptly to the nadir in late winter (2.0 pg/ml) and early spring (4.0 pg/ml). The level then increased gradually until autumn except for a short interruption during early summer.

Serum T_4 levels (Fig. 3) in bucks decreased in late autumn and remained low through the winter. An increase occurred in April, and then the levels dropped again in July and were low through September. The T_4 levels in does displayed a more distinct pattern. The level decreased steadily from mid-autumn to the nadir (4.72 μg/dl) in early spring. It then peaked abruptly (8.51 μg/dl) in mid-spring. The level dropped gradually again until mid-summer (4.66 μg/dl) and remained low until September.

When six periods of equal duration were divided, serum T_4 levels were significantly different among periods. In bucks, the highest levels were found in October–November and the lowest in August–September. In does, the differences were more distinct, with the highest levels in both October–November and April–May and the lowest in July–August. However, there was no significant difference between sexes.

Serum T_3 levels (Fig. 4) in bucks increased from mid to late autumn, then decreased abruptly in early winter. A gradual increase through the winter was followed by a decrease in the spring. The levels increased and peaked (196 pg/dl) in mid-summer, then dropped gradually to the nadir (92 pg/dl) in early autumn. In does, the levels fluctuated throughout the year with the peaks in early winter and mid-summer (225 and 209 pg/dl, respectively), with the lowest levels occurring in late spring and late summer (116 and 121 pg/dl, respectively). T_3 levels were significantly lower during December–January in the bucks. Due to large variations, no significant differences were found in other 2-month durations and between sexes.

Serum C levels (Fig. 5) in both bucks and does fluctuated greatly throughout the year. The nadir in bucks (12.5 ng/dl) and in does (24.2 ng/dl) occurred in

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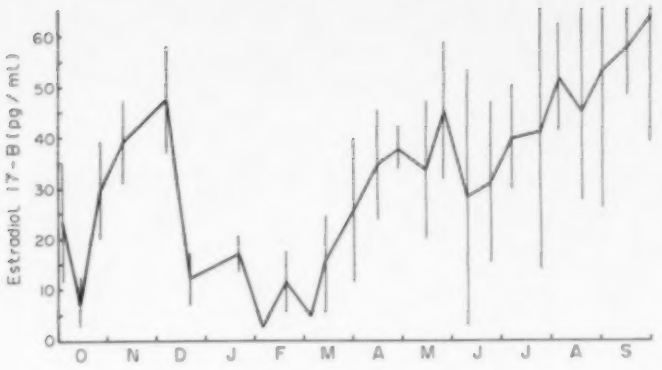

Fig. 2. Annual cycle of serum estradiol 17-β mean ($\pm$ SD) of four female yearling white-tailed deer.

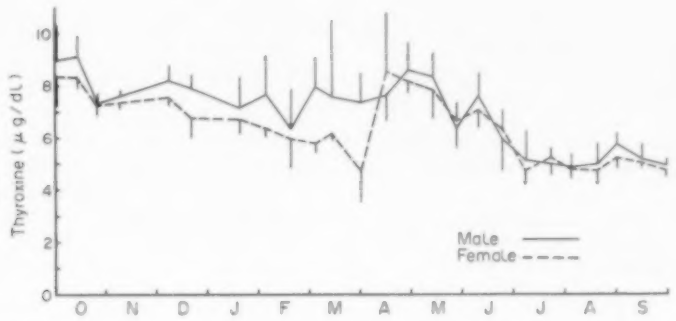

Fig. 3. Annual cycle of serum thyroxine (T_4), mean ($\pm$ SD) of four male and four female yearling white-tailed deer.

late winter. Due to large variations, there were no seasonal and sex differences in serum C levels. However, in most 2-week periods, overall higher C level were found in the female deer (Fig. 5).

Annual cycles in feed intake and body wt

Dry matter intake (g/deer/day) in bucks declined significantly in early and late winter (Fig. 6). An overall decrease of 40% through the winter was followed by a gradual increase through the spring and summer. A slight decrease in late spring was followed by a short-term, abrupt increase (40%) in mid-summer. In does, the pattern of DMI was similar to that found in the bucks with the signifcantly lowest intake in early and late winter and an overall decrease of 50%. A gradual increase in early spring was followed by another drop

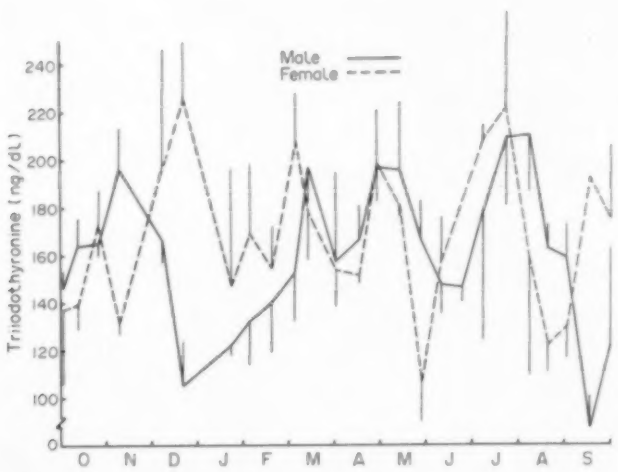

Fig. 4. Annual cycle of serum triiodothyronine (T_3), mean ($\pm$ SD) of four male and four female yearling white-tailed deer.

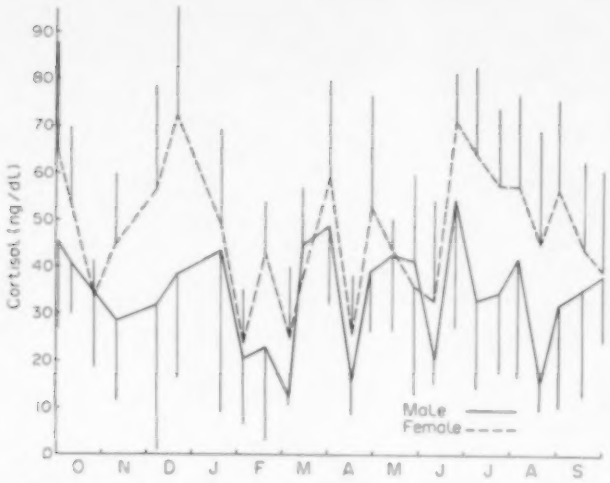

Fig. 5. Annual cycle of serum cortisol, mean (± SD) of four male and four female yearling white-tailed deer.

(30%) in late spring. DMI in December–January and February–March was significantly lower than in other 2-month durations in both sexes.

Body wt in bucks (Fig. 7) decreased slightly to the lowest (39.5 kg) in late winter with a total loss of 14%. A slight increase through the spring was followed by another loss (5%) in May. A gradual increase in the summer was followed by a third drop (8%) in August. In does, body wt increased slightly through the autumn and winter. In late winter an abrupt drop (20%) occurred. Body wt in does decreased slightly to the nadir (34.4 kg) in late spring for a total loss of 11%.

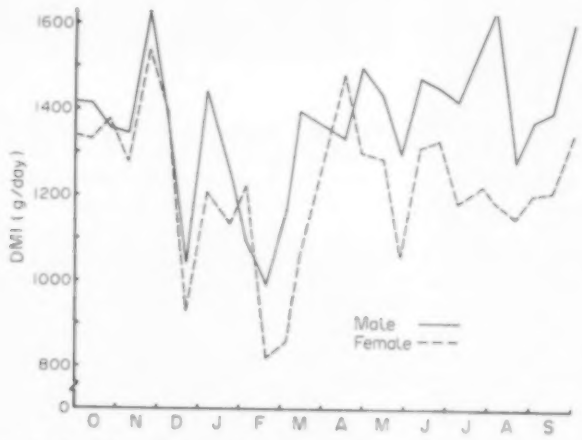

Fig. 6. Dry matter intake of four male and four female yearling white-tailed deer fed a pelleted diet.

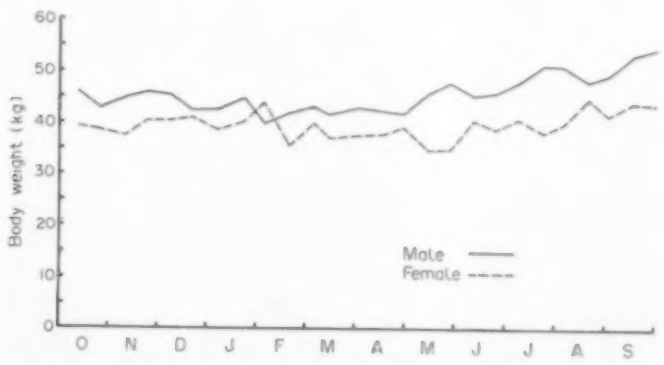

Fig. 7. Body wts of four male and four female white-tailed deer.

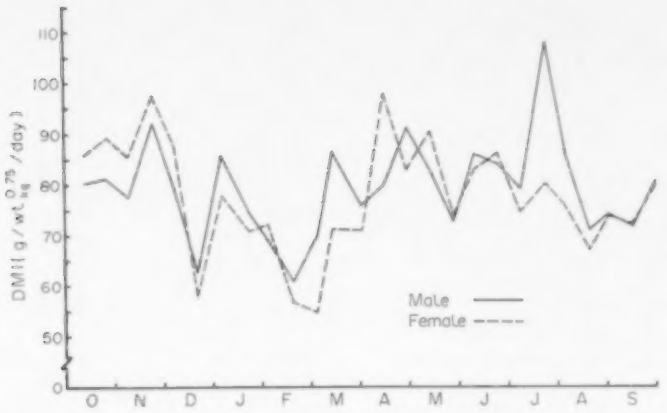

Fig. 8. Dry matter intake (DMI) per unit metabolic body size (body $wt_{kg}^{0.75}$) in four male and four female yearling white-tailed deer fed a pelleted diet.

A gradual increase in the summer was followed by another drop (8%) in late summer. There were four declines in DMI/body $wt_{kg}^{0.75}$ throughout the year in both bucks and does (Fig. 8). Two declines occurred in early winter (December) and late winter (February) with a drop of 30 and 20% respectively in the bucks and 36 and 29% respectively in the does. The other two declines occurred in late spring and mid-summer (17 and 34% in the bucks and 18 and 16% in the does, respectively). An abrupt increase of DMI in mid-summer was followed by the greatest loss (34%) in the bucks.

Correlations between hormones, DMI or body wt and climate data

In bucks, serum T levels were signifcantly correlated with DMI, DL and temperature ($r = -0.42$, -0.69 and -0.67, respectively). Serum T_4 levels were significantly correlated with body wt or temperature ($r = -0.68$ and -0.44, respectively) while T_3 was significantly correlated with DMI and DL ($r = 0.45$ and 0.40, respectively). DMI was significantly correlated with both DL and temperature ($r = 0.51$ and 0.60, respectively), while body wt was correlated with temperature ($r = 0.57$) only. However, in Period 1 during the rut, T levels were significantly correlated with DMI, DL and temperature ($r = -0.82$, -0.73 and -0.76, respectively). In Period 2 after the winter, T_4 and T_3 were significantly correlated with DMI ($r = 0.84$ and 0.86, respectively). During summer heat stress in Period 3, T_4 was significantly correlated with body wt ($r = -0.68$).

In does, serum E levels were significantly correlated with DL and temperature ($r = 0.40$ and 0.54, respectively), while only T_4 was significantly correlated with DMI and body wt ($r = 0.43$ and -0.46, respectively). During the breeding season in Period 1, T_4 correlated significantly with DL and temperature ($r = 0.71$ and 0.86, respectively). However, in Period 2 after the winter, E was significantly correlated with DL and temperature ($r = 0.90$ and 0.79, respectively). T_4 was correlated with DMI and temperature ($r = 0.86$ and 0.76, respectively), while T_3 was correlated with temperature ($r = 0.76$) only. During the summer heat in Period 3, E was significantly correlated with DL and temperature ($r = -0.91$ and -0.77, respectively), while C levels were significantly correlated with body wt ($r = -0.84$).

DISCUSSION

Annual cycles in hormone levels

Serum T levels, which were high during the rut, followed the same pattern as that found in northern states (McMillin *et al.*, 1974; Bubenik *et al.*, 1977), except that they occurred two months later. Serum E levels in the does peaked in early winter and also corresponded with the breeding season. The short-term interruption in the increase of E level during early summer, however, might have been due to initial high ambient temperature and humidity (Thatcher, 1974). The increased levels in the last few months of the study might have been due to increased age and the maturation of the reproductive tract in the does.

The lack of sex differences in T_4 levels was consistent with the study by Bubenik and Bubenik (1978). Seal *et al.* (1972) found that there were no significant differences in T_4 levels throughout the year in northern does with good diets. Bubenik and Bubenik (1978) found no significant differences over the year in mature deer, but they did find significantly higher concentrations in male juveniles. This explains our findings of higher T_4 levels in the initial months and lower levels in the final months of the study as the result of increased age. However, they also may have been due to increasing temperature (Hefco *et al.*, 1975). The T_4 concentrations, ranging from 4.5 to 11.7 μg/dl, were far lower than those found in Canada by Bubenik and Bubenik (1978) (13–24 μg/dl). This could be due to latitude, since in warmer weather rat T_4 concentrations tend to be lower than in colder weather (Hefco *et al.*, 1975).

Winter cold coupled with the breeding season seemed to have a more critical effect on does than bucks, since there was a 20% weight loss in the does and only 14% in the bucks. Hoffman and Robinson's (1966) histological study of thyroid glands in wild white-tailed deer in Maryland showed evidence for the possibility of lowered metabolic activity during winter. During winter, a lowered metabolic rate, as revealed

by lowered T_4 levels, might facilitate not only compensatory rest after the breeding season, but also adaptation to severe winter cold (Bubenik and Bubenik, 1978). The decreased energy intake resulting from winter voluntary hypophagia would result in decreased thyroid activity (Seal *et al.*, 1972). Thus, a primary alteration in appetite, with resulting secondary changes in thyroidal hormone economy, could explain the metabolic adaptations seen in the deer in the northern winter (McMillin *et al.*, 1980). The significant and negative correlation between T_4 and temperature found in our study indicated the existence of an inverse relationship (Hefco *et al.*, 1975). This result contrasts with those from Hoffman and Robinson (1966) and Bubenik and Bubenik (1978) in northern environments. In south Texas, the deer face a less severe winter cold and a more severe summer heat stress (Meyer, 1982). This climate resulted in different responses of deer to the environment in both hormonal changes and hypophagia (Wheaton and Brown, 1983).

In the bucks, T_4 levels were significantly correlated with temperature in Periods 1 and 2, but not in Period 3. This might be due to small sample size, but also it is possible that high temperature in Period 3 interfered with T_4 changes. From a biological viewpoint, it is possible that increased T_4 levels, coupled with increased temperature and DL somewhat in Period 2, provide a better environment for the bucks in initiating antler growth (Bubenik, 1982; Brown *et al.*, 1983). Increased T_4 levels might be a result of, rather than a cause of, increased energy intake (McMillin *et al.*, 1980). The significant correlation between T_4 levels and DMI during this period in males and females ($r = 0.84$ and 0.95, respectively) supports this concept. Results from either lagged temperature or DL provide evidence that serum T_4 levels are both temperature and DL dependent in the bucks. In the does, the lack of a significant correlation of T_4 with either DL or temperature indicates that T_4 changes may be independent of both temperature and DL. However, in late May as temperature increased to 27°C, the significant drop of T_4 levels in both male and female deer confirms the study of Hefco *et al.* (1975) that the fall of T_4 production in the heat is the direct consequence of an elevated temperature. It seems that a short-term (1-week) effect of temperature on serum T_4 levels exists in deer.

Serum T_3 levels are directly dependent on energy intake (Seal *et al.*, 1978). This was true in our study, especially during late winter and late spring. This might be due to the effect of increased DMI (McMillin *et al.*, 1980). Increased DL and temperature may be the mover for increased DMI and in turn resulted in increased T_3 levels during Period 2. Results from lagged DL showed that it played a more important role in serum T_3 changes in bucks. The lack of significant correlations between serum T_3 and any of the climatic parameters indicated that serum T_3 levels in does might be independent of DL and temperature. It is also possible that individual variations mask a significant correlation between serum T_3 and DL or temperature. However, Bahanak *et al.* (1981) suggested that although total serum T_3 and T_4 concentrations in does did not always correlate well with the degree of thyroid gland activity, they were still good indicators of nutritional stress. In our findings, serum T_4 and T_3 in the bucks and T_4 in the does were good indicators for studying hypophagia if coupled with gonadal hormone changes.

The lack of sex and seasonal differences in C levels agrees with the findings of Seal *et al.* (1972) and Bubenik *et al.* (1975a). The large variations within groups agree with the finding of Bubenik *et al.* (1975a) of large variations in C levels between individual deer.

Annual cycles of feed intake and body wt

Significantly lowered DMI in December and February indicated that winter, coupled with rutting season, had a significant effect on DMI in South Texas white-tails (Wheaton and Brown 1983). Cowan (1971) attributed the short-term intake suppression during mid-summer to the association with hormonal changes triggered by the shortened days after the summer solstice. Wheaton and Brown (1983) and Holter *et al.* (1977) attributed this to warm and humid weather. In August–September all deer had heavier body wts than in February–March or April–May. The increased body wt during the final months could be the result of increased age, but the significantly lowered body wt during late winter could be the result of winter cold coupled with rutting activities. Early summer weight loss (17%) in bucks and late spring weight loss (10%) in does, which agree with findings by Short *et al.* (1968), may have been due to the effects of initial short-term high ambient temperatures.

Interrelationships among hormone levels, DMI, body wt and climate

The negative correlation between serum T level and DMI, especially during the rut (December–January) confirms the hypothesis offered by McMillin *et al.* (1980) that fall hypophagia is directly under the control of the annual T concentrations in bucks, and that high concentrations of T trigger a behavioral response of appetite suppression in the hypthalamus. This is consistent also with Mazur's (1974) findings that there was an inverse relationship between intake and circulating plasma T concentrations in male deer during the rut and winter, but that the relationship was less clear in the spring and summer.

Daylength has its effects primarily by changing the sensitivity of the hypothalamus to circulating steroids in red deer stags (*Cervus elaphus*) (Lincoln and Kay, 1979). Also Mirarchi *et al.* (1977) pointed out that decreasing DL was associated with increased pituitary LH secretion, causing a rise in circulating LH levels which in turn stimulated the interstitial cells of Leydig to produce T. Studies by McMillin *et al.* (1974) suggested that the T cycle was an inherent circannual rhythm which could be synchronized to the photoperiod. They suggested, however, that under constant, unequal daily periods of light and dark, the approximately yearly T cycles were independent of nutrition and temperature changes. It seems that in our study T levels are dependent on both DL and ambient temperature changes.

The lack of a significant correlation between serum E levels and DMI indicated that the breeding season in does seemed to have a less distinct effect on hypophagia than T levels did in bucks. According to Wade's

(1972) study in rats, E is the principal ovarian steroid regulating body wt in females and acts on separate neural loci to inhibit DMI. There was no such relationship found in the deer in our study. Serum E levels increased along with increased DL and temperature, especially in Period 2. In Period 3, as temperatures were usually greater than 27°C, the negative correlations between E levels and both DL and temperature may have been due to the suppression of E from high ambient temperatures and humidity or due to the effects of DL changing from increasing to decreasing. Correlations of lagged temperature and DL up to 7 weeks with E indicated that both had overall long-term effects on serum E levels.

The difficulty in studying blood C levels was reported by Bubenik *et al.* (1977), in which an elevation of blood C concentrations as much as 350% in white-tailed deer was found in just 15 min. However, in attempting to avoid the sudden increase of C levels in our study, Rompun was used as tranquilizer to facilitate bleeding without causing a significant stress. The large variation in this study was not due to sudden seasonal fluctuations, but due to individual differences (i.e. some animals had overall high C concentrations while others had lower concentrations throughout the year). It is possible that large individual variations mask a clear seasonal trend and any correlation with other parameters (Bubenik *et al.*, 1977).

During and after the rut, significant correlation of DMI with DL supported the studies by French *et al.* (1960), Cowan (1971) and Simpson and Suttie (1983). Initial high temperature (27°C), however, had a significant short-term effect on DMI in bucks and does, i.e. in early summer. According to the study by McDowell *et al.* (1969) in cattle, during the first week of elevated temperatures, the decreased DMI might be the result of the effect on the appetite center and the tendency to let a high respiration rate interfere with feeding. Conrad (1966) also pointed out that thermostatic mechanisms may dominate regulation of feed intake independently of other factors. Thompson *et al.* (1963) found that adverse effects of the hot environment became conspicuous soon after the initial heat exposure in dairy heifers. Our study in deer showed the same result; the suppression of DMI by initial high ambient temperature was apparent.

In bucks, body wt was significantly correlated with temperature. Results from lagged temperature also provided evidence that temperature had both short- and long-term effects on body wt in bucks. In does, the climatic effects on body wt were less clear than those in bucks. However, winter cold caused a 20% loss in body wt in a short period, and the initiation of high temperature in late spring caused a 12% body wt loss. It seems that short-term climatic effects were clear in the does.

In conclusion, the hypophagia found in winter in both male and female South Texas white-tailed deer is no doubt an endogenous rhythm synchronized by environmental factors and the breeding season. Winter cold coupled with the breeding season and of initial and prolonged heat in late spring and summer result in a unique form of hypophagia somewhat different from that found in the Northern states. Due to the number of animals in this study, the results cannot be considered conclusive. Nonetheless it is evident that the thyroid hormones along with the sex steroids can function as indicators of nutritional condition, and may play a role as the link between environmental cues and feed intake in deer.

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STRUCTURE AND COMPOSITION OF THE EGGS FROM SEVERAL AVIAN SPECIES

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Abstract—1. There is a remarkable uniformity in both albumen and yolk density as well as in the proportion of the egg yolk with respect to egg weight.

2. There are large variations in the concentration of minor components of egg such as free amino acids and carbohydrate.

3. Most of egg phosphate is in the yolk in the form of phospholipids with a significant proportion as phosphoproteins.

4. Most chemical parameters studied had less variation in yolk than in albumen. In general the composition of yolk is more conservative despite egg size and taxonomic situation of the bird.

INTRODUCTION

The avian egg, as a closed system that provides all foodstuffs needed for embryo development, has been widely studied, as a biological system (Freeman and Vince, 1974) as well as a component of human diet (Rose and Vahlteich, 1938; Everson and Souders, 1957; Parkinson, 1966). Its composition in the domestic fowl is of important economic interest, as are the factors that can affect their size, composition and viability. Because of this importance most studies on eggs have been carried on poultry because of the availability of animals and for the direct implication of the results. However, the nutritional use of other avian eggs has been receiving some attention in the literature (Lahiri and Baliga, 1970).

We have studied the variation of composition parameters in eggs of domestic fowl (Sáinz *et al.*, 1983) as well as some components of a wide range of birds eggs (Roca *et al.*, 1982), correlating some of their constituents with known facets of their behavior and development.

The influences of external (Weiss *et al.*, 1964; Cruickshank, 1941; Cunningham *et al.*, 1960) as well as internal or taxonomically-linked and genetic (Edwards, 1964; Washburn, 1979) factors on avian embryo development and also in egg composition have been largely studied using mostly domestic fowl eggs, the literature available for other species being extremely scarce (Lahiri and Baliga, 1970; Christie and Moore, 1972). We have intended here to study in a wide spectrum of available species of birds from the island of Majorca (both farm-raised and wild) some parameters of the composition of their eggs as a first step in the discrimination of variable components and those other materials which proportion and availability are conservative in the evolution of egg because of their criticality in the embryo development.

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MATERIALS AND METHODS

Recently laid unincubated eggs from wild nests of quail (*Coturnix coturnix*), redlegged partridge (*Alectoris rufa*), house sparrow (*Passer domesticus*) and snipe (*Gallinago gallinago*) were obtained in different places of the island of Majorca. Unincubated eggs from farm-raised domestic fowl (*Gallus domesticus*, Rhode Island red), turtle dove (*Streptopelia turtur*), mallard (*Anas platyrhynchos*), muteduck (*Anas moschata*), goose (*Anser anser*), rock dove (*Columba livia*), turkey (*Meleagris gallopavo*), guinea fowl (*Numida meleagris*), golden pheasant (*Chrysolopus pictus*) and pheasant (*Phasianus colchicus*) were collected on the S'Avall estate on Majorca. In all cases only one egg was used from each nest. Domestic fowl, mute duck, turkey, rock dove and guinea fowl were fed with grain and kitchen waste; the other animals had a free natural alimentation.

The eggs were weighed and dissected for albumen, yolk, shell and membranes. Egg yolk, white and shell were weighed free of membranes, and their densities were measured by their floatability in known density mixtures of xylene and chloroform in a thermostatic system (Roca *et al.*, 1982). Egg yolk and albumen were used for lipid extraction and purification (Folch *et al.*, 1957), as well as for deproteinization with cold acetone (Arola *et al.*, 1977), total protein (Rheinhold, 1961) determination, and water content estimation through differential weighing after dessication in an oven at 105°C. Clear acetone supernatants were used for the valoration of free glucids (Pons *et al.*, 1981) and amino acids (Allen *et al.*, 1977). Acetone precipitates were digested for 10 min in 2 N NaOH. After neutralization with 6 N HCl, denatured protein was precipitated with cold trichloroacetic acid to a final 10% concentration, the supernatant being used for carbohydrate estimation (Pons *et al.*, 1981). Shell density was estimated by differential weighing first in water and then in ethanol, after boiling the samples for 15 min in ethanol in order to exclude all air from the pores. The differences in volume were considered to be due to air in the shell pores and the percent of air in the shell was calculated.

Yolk, albumen, TCA-precipitated protein and extracted lipid samples of both fractions were used for hot sulphuric acid mineralization (Sáinz *et al.*, 1983) and inorganic phosphate estimation (Power, 1961).

RESULTS AND DISCUSSION

In Table 1 the weights and densities of eggs and their main fractions: shell albumen and yolk, together with shell thickness and pore volume, are presented. The range of weights of the eggs studied was considerable; from that of house sparrow to that of goose the differences of weight spanned over a 55-fold difference. Albumen weight was remarkably constant expressed as a percent of egg weight, with a maximal difference of 38% between rock dove and guinea fowl. The uniformity in yolk size was also considerable, with a maximal range of 83%, and that of shell weight even greater, with comparatively heavier shells in guinea fowl and lighter for doves and quail (a 150% variation overall).

Despite these differences, albumen density remained very much uniform, with an overall variation of less than 1.6%, and that of yolk even more constant: 0.9%. Shell thickness had ample variation because of the combined effects of shell proportional weight and egg size, being minimal for house sparrow and thicker for goose. However, the range of thickness was of about 330%, while the differences of weights were actually two orders of magnitude apart and shell proportional weight had a comparable variability. Shell pore volume showed a considerable variation (about 26-fold in the sample studied); this can be due to the combination of both an effect of larger or smaller egg size and also the type of pore found in the egg surface. Shell pores are necessary for gas exchange of the developing embryo (Board and Scott, 1980) and can be classified according to shape and structure, the anatidae, columbidae and gallinaceae, in general terms, belonging to different types (Board *et al.*, 1977).

In Table 2 the water in both albumen and yolk, together with protein and free amino acids in these fractions are presented. Water in albumen and yolk showed actually little variation (5% and 38% of maximal range respectively), in accordance with the conservative and critical importance of water content for the avian embryo development (Ar and Rahn, 1980). Water content of yolk was on the whole much lower than that of albumen because of the important lipid content of the first. Protein content was also about twice higher in yolk than in albumen, with little variation between the groups (a twofold and 1.5-fold range respectively for albumen and yolk) despite important differences in individual protein compo-

Table 1. Weights, densities and other parameter of the unincubated eggs from several species of birds

Species	N	Egg weight (g)	Albumen weight (%cw)	Yolk weight (%cw)	Shell weight (%cw)	Albumen density (g/ml)	Yolk density (g/ml)	Shell pore volume (%)	Shell thickness (mm)
Numida meleagris	5	36.9 ± 1.2	47.3 ± 2.4	33.6 ± 1.4	15.3 ± 0.1	1.039 ± 0.001	1.025 ± 0.001	0.92 ± 0.10	0.42 ± 0.01
Coturnix coturnix	5	8.5 ± 0.1	54.0 ± 1.0	31.6 ± 1.0	6.7 ± 0.2	1.041 ± 0.001	1.030 ± 0.002	0.47 ± 0.06	0.15 ± 0.00
Gallus domesticus	5	56.5 ± 2.5	57.2 ± 0.7	30.3 ± 0.4	9.7 ± 0.4	1.036 ± 0.001	1.023 ± 0.000	1.47 ± 0.21	0.34 ± 0.01
Alectoris rufa	5	17.7 ± 1.2	48.5 ± 2.0	34.7 ± 0.6	10.4 ± 0.5	1.030 ± 0.000	1.024 ± 0.000	1.39 ± 0.13	0.25 ± 0.00
Chrysolophus pictus	3	20.4 ± 2.4	47.1 ± 3.1	38.6 ± 3.4	10.2 ± 0.7	1.033 ± 0.001	1.025 ± 0.002	1.74 ± 0.06	0.26 ± 0.00
Phasianus colchicus	5	30.2 ± 0.6	53.4 ± 0.4	31.3 ± 0.4	9.8 ± 0.4	1.035 ± 0.002	1.024 ± 0.002	1.91 ± 0.28	0.26 ± 0.01
Meleagris gallopavo	5	84.4 ± 2.7	54.0 ± 1.9	32.5 ± 1.6	8.4 ± 0.2	1.039 ± 0.001	1.021 ± 0.001	1.55 ± 0.13	0.24 ± 0.01
Columba livia	5	17.5 ± 1.8	65.0 ± 1.8	21.1 ± 1.5	7.1 ± 0.3	1.032 ± 0.001	1.027 ± 0.001	0.24 ± 0.03	0.20 ± 0.01
Streptopelia turtur	2	7.5 ± 0.4	59.8 ± 1.5	27.5 ± 0.6	6.1 ± 0.2	1.036 ± 0.001	1.022 ± 0.001	0.18 ± 0.00	0.13 ± 0.00
Gallinago gallinago	4	7.3 ± 0.1	5.28 ± 1.1	35.5 ± 0.8	7.6 ± 0.2	1.035 ± 0.000	1.021 ± 0.001	0.45 ± 0.03	0.15 ± 0.01
Anas moschata	5	58.9 ± 3.0	51.5 ± 1.5	31.2 ± 1.9	12.6 ± 0.3	1.037 ± 0.002	1.024 ± 0.002	2.01 ± 0.18	0.47 ± 0.01
Anas platyrhynchos	5	58.2 ± 1.4	51.3 ± 0.9	34.5 ± 0.8	8.9 ± 0.2	1.031 ± 0.001	1.023 ± 0.001	1.59 ± 0.25	0.31 ± 0.01
Anser anser	5	153.6 ± 1.7	54.9 ± 0.2	29.4 ± 0.3	12.8 ± 0.1	1.035 ± 0.000	1.032 ± 0.001	2.60 ± 0.23	0.57 ± 0.01
Passer domesticus	5	2.8 ± 0.1	62.2 ± 2.1	23.6 ± 1.3	12.6 ± 0.9	1.025 ± 0.000	1.026 ± 0.000	0.10 ± 0.01	0.11 ± 0.00

Data are the mean ± SEM; cw = egg weight.

Table 2. Water, free amino acids and protein content of the unincubated eggs from several species of birds

Species	N	Albumen water (%)	Yolk water (%)	Albumen protein (mg/g)	Yolk protein (mg/g)	Albumen amino acids (μmol/g)	Yolk amino acids (μmol/g)
Numida meleagris	5	90.1 ± 0.3	50.3 ± 1.7	81.1 ± 4.7	158 ± 4	3.46 ± 0.20	43.2 ± 0.8
Coturnix coturnix	5	87.8 ± 0.6	46.7 ± 2.6	83.3 ± 5.0	227 ± 13	3.56 ± 0.20	36.6 ± 0.9
Gallus domesticus	5	90.6 ± 0.5	48.4 ± 0.6	76.0 ± 7.2	185 ± 5	0.90 ± 0.07	33.9 ± 1.5
Alectoris rufa	5	90.7 ± 1.0	49.4 ± 1.0	80.9 ± 8.4	185 ± 4	4.26 ± 0.21	24.5 ± 1.1
Chrysolopus pictus	3	90.3 ± 0.9	49.9 ± 1.5	86.3 ± 6.6	200 ± 5	3.70 ± 0.63	49.7 ± 3.6
Phasianus colchicus	5	89.0 ± 0.9	47.5 ± 0.7	91.3 ± 9.7	206 ± 3	3.09 ± 0.02	49.7 ± 1.8
Meleagris gallopavo	5	89.0 ± 0.6	46.9 ± 0.8	83.2 ± 4.5	141 ± 4	2.18 ± 0.40	32.8 ± 0.9
Columba livia	5	90.0 ± 0.6	56.6 ± 2.0	61.6 ± 5.4	161 ± 3	1.32 ± 0.13	19.2 ± 1.1
Streptopelia turtur	2	90.8 ± 0.1	61.5 ± 0.2	65.0 ± 8.2	140 ± 5	1.34 ± 0.16	25.4 ± 3.1
Gallinago gallinago	4	88.2 ± 0.6	46.0 ± 1.1	79.5 ± 4.8	157 ± 4	1.56 ± 0.05	22.0 ± 1.3
Anas moschata	5	89.0 ± 1.1	46.6 ± 1.0	76.3 ± 6.4	164 ± 4	5.80 ± 0.55	45.1 ± 1.0
Anas platyrhynchos	5	90.0 ± 0.4	46.2 ± 1.5	85.8 ± 3.8	179 ± 5	6.62 ± 0.35	35.0 ± 1.2
Anser anser	5	86.6 ± 0.4	44.5 ± 0.6	110.2 ± 5.2	188 ± 7	3.10 ± 0.04	50.0 ± 1.2
Passer domesticus	5	89.2 ± 0.6	56.4 ± 3.5	56.5 ± 2.0	182 ± 9	5.32 ± 0.25	35.9 ± 0.8

Data are the mean ± SEM; all weights refer to that of the fraction.

Table 3. Free and protein-bound carbohydrate and lipid content of the unincubated eggs from several species of birds

Species	N	Albumen free glucid (μmol/g)	Yolk free glucid (μmol/g)	Albumen protein glucid (%)	Yolk protein glucid (%)	Albumen lipids (mg/g)	Yolk lipids (mg/g)
Numida meleagris	5	11.4 ± 1.4	6.5 ± 0.4	1.73 ± 0.06	0.79 ± 0.03	6.34 ± 1.81	348 ± 7
Coturnix coturnix	5	16.3 ± 1.6	7.1 ± 0.7	1.58 ± 0.07	0.71 ± 0.02	1.42 ± 0.10	336 ± 10
Gallus domesticus	5	14.9 ± 1.4	7.2 ± 0.2	1.38 ± 0.05	0.64 ± 0.02	0.42 ± 0.07	352 ± 8
Alectoris rufa	5	22.7 ± 1.4	4.8 ± 0.7	1.48 ± 0.01	0.76 ± 0.01	1.63 ± 0.29	335 ± 15
Chrysolopus pictus	3	14.8 ± 0.7	7.5 ± 1.7	1.54 ± 0.15	0.87 ± 0.02	8.13 ± 0.97	372 ± 6
Phasianus colchicus	5	20.9 ± 1.2	3.2 ± 0.2	1.47 ± 0.03	0.61 ± 0.02	3.64 ± 0.19	371 ± 9
Meleagris gallopavo	5	12.7 ± 1.6	5.0 ± 0.5	1.57 ± 0.09	0.64 ± 0.00	1.40 ± 0.30	359 ± 5
Columba livia	5	6.9 ± 0.6	9.1 ± 1.0	3.74 ± 0.03	0.96 ± 0.04	1.98 ± 0.32	265 ± 13
Streptopelia turtur	2	4.0 ± 0.7	2.7 ± 0.4	1.62 ± 0.03	1.44 ± 0.29	1.35 ± 0.15	279 ± 4
Anas moschata	5	10.2 ± 1.7	7.9 ± 0.2	1.43 ± 0.07	0.73 ± 0.02	3.20 ± 0.58	372 ± 12
Anas platyrhynchos	5	14.1 ± 4.0	7.4 ± 0.3	1.51 ± 0.06	0.63 ± 0.01	5.08 ± 1.18	376 ± 6
Anser anser	5	14.1 ± 4.1	2.3 ± 0.2	1.82 ± 0.05	0.89 ± 0.03	4.16 ± 0.72	352 ± 5
Passer domesticus	5	12.2 ± 1.3	9.3 ± 1.2	3.00 ± 0.02	1.10 ± 0.02	2.92 ± 0.19	280 ± 15
Gallinago gallinago	4	6.1 ± 0.5	4.1 ± 1.7	2.17 ± 0.01	0.60 ± 0.02	0.95 ± 0.15	359 ± 7

Data are the mean ± SEM; free glucid concentrations refer to μmol of hexosyl residues equivalent.

sition of the eggs (Fevold, 1951) of different species (Bain and Deutsch, 1947). The results found agreed with the available data on hens mainly (Rose and Vahlteich, 1938; Everson and Souders, 1957; Lahiry and Baliga, 1970). Maximal albumen protein was found in the goose and minimal in the house sparrow and the pigeons; in the yolk, galliforms had the highest protein values, being the lowest for snipes.

Free amino acids show a wide range of variation in both albumen and yolk, with much higher values in the yolk, and concentration ratios between yolk and albumen ranging from 38 in the domestic fowl to only 5.3 in the mallard. The overall variation in the albumen was of 640%, and 130% in the yolk. The amount of free amino acids is, however, much smaller than that of peptide amino acids (Lunven *et al.*, 1973), despite their fast utilization during initial stages of development (Williams *et al.*, 1954).

In Table 3 the carbohydrate and lipid content of the eggs studied are presented. The free glucide fraction corresponds mainly to noncovalently bound glucids—mainly glucose—to protein (García *et al.*, 1982) produced by direct reaction in the egg between carbohydrate and the proteins (Feeney *et al.*, 1964), while the alkaline-removed carbohydrate corresponded mainly to glycoprotein glucide. The differences between both parameters were, however, not very clear because of methodological problems in the definition of protein-linked carbohydrate in the egg (García *et al.*, 1982). The variation in these glucidic components of the egg was relatively small, more conservative in the yolk than in the albumen with very low "free" carbohydrate levels compared with those released by alkaline treatment.

Lipid content of albumen varied considerably, even between closely related species such as the two pheasants. In general, the differences were much more marked than those found in intraspecific studies (Sáinz *et al.*, 1983) despite the wide variation of this parameter. Yolk lipids, however, had a maximal dispersion of 40% in the groups studied and ranged between one fourth to one third of all yolk weight. This uniformity agreed with the little variation found in egg lipids by changes in nutrition or other factors (Edwards, 1964; Weiss *et al.*, 1964), being mainly constituted by triacylglycerols (Christie and Moore, 1972) and significant amounts of cholesterol (Froning, 1971) and phospholipids (Christie and Moore, 1972).

The distribution in fractions of egg phosphate can be seen in Table 4. Total albumen phosphate showed ample variations, probably fueled by the very low amounts of phosphate in this fraction, a considerable

Table 4. Phosphate content and distribution in the unincubated eggs from several species of birds

Species	N	Albumen total phosphate (μmol/g)	Yolk total phosphate (μmol/g)	Albumen protein phosphate (μmol/g)	Yolk protein phosphate (μmol/g)	Albumen protein phosphate (mg/g*)	Yolk protein phosphate (g/100 g*)	Yolk lipid phosphate (μmol/g*)	Yolk lipid phosphate (mg/g*)
Numida meleagris	5	2.05 ± 0.13	158 ± 7	0.81 ± 0.10	54.1 ± 3.9	1.05 ± 0.11	3.34 ± 0.28	103.5 ± 3.6	27.7 ± 2.1
Coturnix coturnix	5	3.84 ± 0.35	158 ± 9	0.69 ± 0.04	44.5 ± 0.8	0.83 ± 0.09	1.99 ± 0.13	113.0 ± 8.9	32.8 ± 2.0
Gallus domesticus	5	1.27 ± 0.09	151 ± 1	0.34 ± 0.00	53.7 ± 0.8	0.44 ± 0.03	2.82 ± 0.05	97.2 ± 1.4	28.1 ± 1.8
Alectoris rufa	5	1.52 ± 0.19	158 ± 3	0.25 ± 0.02	63.2 ± 1.0	0.31 ± 0.04	3.32 ± 0.12	94.7 ± 2.3	27.7 ± 0.8
Chrysolopus pictus	3	5.42 ± 0.35	128 ± 4	0.31 ± 0.00	54.1 ± 1.3	0.41 ± 0.04	2.63 ± 0.01	73.7 ± 3.8	20.4 ± 1.4
Phasianus colchicus	5	2.43 ± 0.63	124 ± 2	0.41 ± 0.01	44.2 ± 0.8	0.46 ± 0.06	2.08 ± 0.04	79.8 ± 1.2	21.2 ± 0.6
Meleagris gallopavo	5	2.14 ± 0.18	146 ± 4	0.77 ± 0.02	54.3 ± 2.2	0.90 ± 0.04	3.75 ± 0.11	91.8 ± 3.0	25.0 ± 1.0
Columba livia	5	6.82 ± 0.26	134 ± 5	1.96 ± 0.21	51.6 ± 1.9	3.18 ± 0.40	3.11 ± 0.08	72.0 ± 3.0	26.8 ± 1.7
Streptopelia turtur	2	5.55 ± 0.95	125 ± 1	0.76 ± 0.07	49.1 ± 1.5	1.18 ± 0.23	3.41 ± 0.22	75.5 ± 0.5	26.5 ± 0.2
Gallinago gallinago	4	2.75 ± 0.02	136 ± 3	0.44 ± 0.01	53.7 ± 2.5	0.57 ± 0.09	3.04 ± 0.11	82.5 ± 1.5	22.6 ± 0.7
Anas moschata	5	1.05 ± 0.07	158 ± 2	0.30 ± 0.01	56.2 ± 1.5	0.34 ± 0.04	3.36 ± 0.08	102.2 ± 11.9	26.8 ± 2.5
Anas platyrhynchos	5	1.44 ± 0.06	130 ± 3	0.43 ± 0.04	48.4 ± 3.7	0.48 ± 0.05	2.62 ± 0.72	80.6 ± 2.7	21.0 ± 0.5
Anser anser	5	1.32 ± 0.06	143 ± 4	0.21 ± 0.01	60.4 ± 2.9	0.19 ± 0.10	3.17 ± 0.12	82.6 ± 2.0	23.0 ± 0.7
Passer domesticus	5	9.49 ± 1.12	149 ± 7	1.66 ± 0.46	53.6 ± 2.6	0.88 ± 0.29	2.88 ± 0.17	95.4 ± 7.2	33.2 ± 1.8

Data are the mean ± SEM; * = per unit of protein or lipid weight.

part of it being constituted by protein phosphate as indicated in the third column (Fevold, 1951; Parkinson, 1966). Most of the egg phosphate is found in the yolk (Everson and Souders, 1957; Froning, 1971), both linked to phosphoproteins (Fevold, 1951) and in the form of phospholipids (Rhodes and Lea, 1956).

The range of variation of yolk total lipids was very small, 27% being the rather uniform proportion of protein phosphate (40% variation). When the phosphate data were expressed per unit of protein or lipid weight, the variations in albumen phosphoprotein widened but those of the yolk remained constant, and the phospholipid proportion to total lipid was also constant (60% variation).

The data presented agree with a remarkable uniformity of some physical and structural features of the shell egg, such as the proportion of albumen and yolk, and their densities. The chemical composition of the egg was more uniform in the yolk than in the albumen, with uniform concentrations of phosphorous and lipidic materials, constant protein and water and widely changing proportions of glucide material (Svensson, 1964; Roca *et al.*, 1982) and free amino acids. This can be partly due to the utilization of these materials in the initial stages of avian embryo development (Yarnell *et al.*, 1966; Freeman and Vince, 1974) and to methodological problems (García *et al.*, 1982) in the estimation of these small components of the egg. The uniformity of yolk composition stands despite wide differences in size, structure and species of the eggs studied.

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THE EFFECT OF ENVIRONMENTAL AND ENDOGENOUS FACTORS ON BLOOD CONSTITUENTS OF RAINBOW TROUT (*SALMO GAIRDNERI*)—I. FOOD CONTENT OF STOMACH AND INTESTINE

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Abstract—1. During a 5-day lack of food the liver weight of rainbow trout decreased.
2. The food content of stomach and intestine had maximal levels at 1–4 hr after the last feeding, followed by an asymptotic reduction during starvation.
3. The plasma sodium and potassium concentration correlated negatively with the gastric and intestinal content, which could be explained by the physiological processes of digestion.
4. Plasma glucose, lipid, alkaline phosphatase and α-amylase were affected by the gastric and intestinal food content.

INTRODUCTION

Many fish species tolerate long periods of starvation, the physiological consequences of which are well documented in the blood (Love, 1970; Sauer and Haider, 1979). However, only sparse data exist on the effect of short term lack of food on rainbow trout blood (Bergot, 1979a, b). That is the premise to standardize the space of time between feeding and blood withdrawal in hematological experiments. In this study the quantitative changes of 22 blood constituents were recorded during 5 days of hunger.

METHODS

Twenty-five rainbow trout at the age of 2.5 yr were used in the first experiment (group I), and 50 trout at the age of 10 months in the second one (group II). The fish adapted 35 days to running water (2 l/sec, 11–12°C) in 200 l. aquaria, feeding on commercial 3 mm pellets twice a day. The daily ration—adjusted to fish size and water temperature—amounted to 1.5% (group I) and 2.0% (group II) of the body weight. Group I was sampled 1, 4, 16, 40 and 72 hr after the cessation of feeding. In experiment II blood was withdrawn 4, 16, 40, 64, 88 and 112 hr after the last feeding. The fish were separately and cautiously netted, killed by electroshock (220 V, 1 min), the heart was punctured (Lehmann and Stürenberg, 1974, 1980), and the blood withdrawn into heparinized tubes. After the determination of hematocrit (Snieszko, 1960) and hemoglobin (hemoglobincyanid method, Merckotest set) the plasma was separated by centrifugation and the following plasma constituents were analyzed with Merckotest sets: GOT = glutamate–oxalacetate–transaminase, GPT = glutamate–pyruvate–transaminase, LDH = lactatedehydrogenase, HBDH = hydroxybutyratedehydrogenase = isoenzyme 1 and 2 of LDH (opt. u.v.-test); α-amylase (Street and Close); AP = alkaline phosphatase (Bessey *et al.*); LAP = leucin-arylamidase (EC 3.4.1.2, substrate L-leucin-4-nitroanilid); chloride (mercurimetric); cholesterol (Liebermann and Burchard); creatinine (picric acid reaction); glucose (GOD-PAP-method); anorganic phosphate and phosphatides (molybden blue-method); total lipid (sulphophospho-vanilline-reaction); total protein (biuret); urea (Berthelot). The plasma electrolytes sodium, potassium, calcium and magnesium were analyzed with atom absorption spectroscopy. The food content of stomach and intestine was weight together, and the liver weight recorded (for further details of the methods see Hille and v. Pöllnitz, 1980).

RESULTS

The liver weight of hungry trout decreased; on the fifth day it amounted only to 57% of the 4 hr value Fig. 1). The gastric and intestinal food content attained maximal levels at 1–4 hr after feeding; it decreased after 16–40 hr significantly, the stomachs of all trout being completely empty at 16 hr (Fig. 1). In contrast, plasma sodium and chloride had the lowest concentrations at 4 hr after feeding and increased in the blood of hungry trout (Fig. 2). Therefore, a negative correlation existed between the plasma electrolyte content and the amount of undigested food (Table 1).

Plasma glucose had maximum concentrations at 4 hr after feeding and decreased afterwards as well as the lipid content until 16–40 hr, when both parameters adjusted to constant levels (Fig. 3). Only AP and α-amylase decreased further (Fig. 4). Magnesium, protein, urea and phosphate varied inconsistently only in some samples. The other blood constituents did not change significantly.

DISCUSSION

The reduction of the liver weight of hungry trout derives from the function of this organ, acting as the first source of energy compounds in hunger (Shimma *et al.*, 1976a; Heidinger and Crawford, 1977; Abel *et al.*, 1979; Milne *et al.*, 1979).

The stomach depletion of group II (small trout, completed after 16 hr) is in accordance with the data of Grove *et al.* (1978). The size dependence of the stomach depletion, observed by Grove *et al.* (1978), did not exist

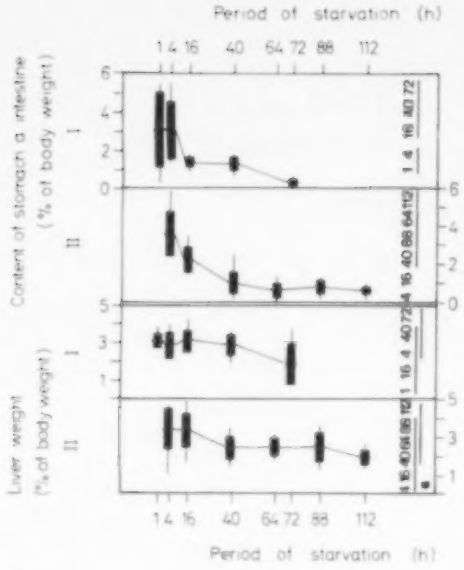

Fig. 1. Changes in the content of the alimentary canal and the liver weight during hunger in two groups of rainbow trout (I and II).

Blocks are standard errors, small vertical lines show extreme values, the means are connected by the small horizontal lines. Number of fish per sample: 5 in group I, 8 in group II. Statistics: Duncan's new multiple-range test. Sample means (indicated by sample time after the last food intake) not significantly from each other are underlined. In general $P < 0.01$. *$P < 0.05$.

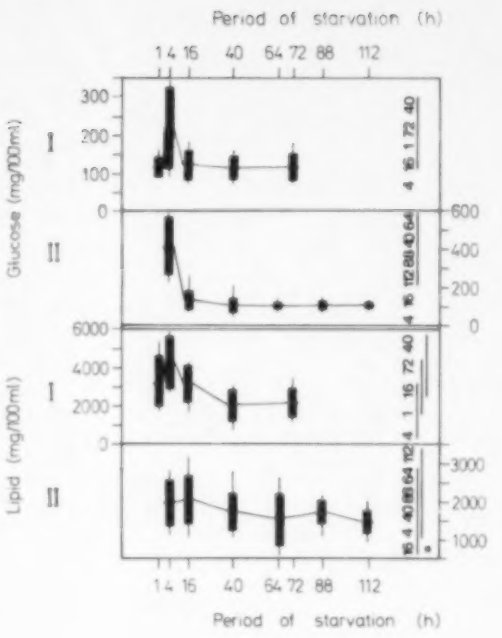

Fig. 3. Changes in plasma lipid and glucose concentrations during hunger in two groups of rainbow trout (I and II). Explanations, see Fig. 1.

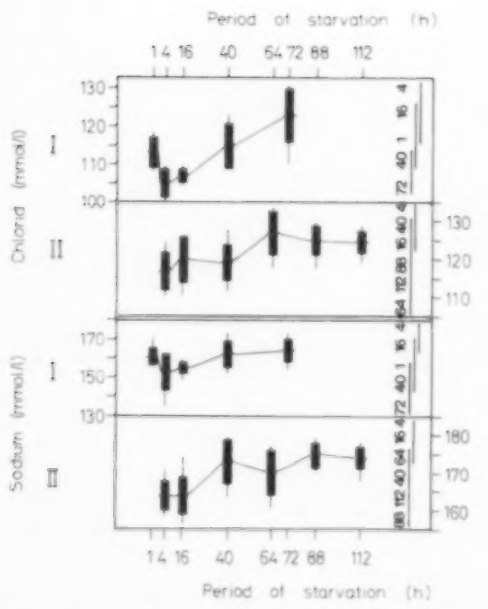

Fig. 2. Changes in plasma sodium and chloride concentrations during hunger in two groups of rainbow trout (I and II). Explanations, see Fig. 1.

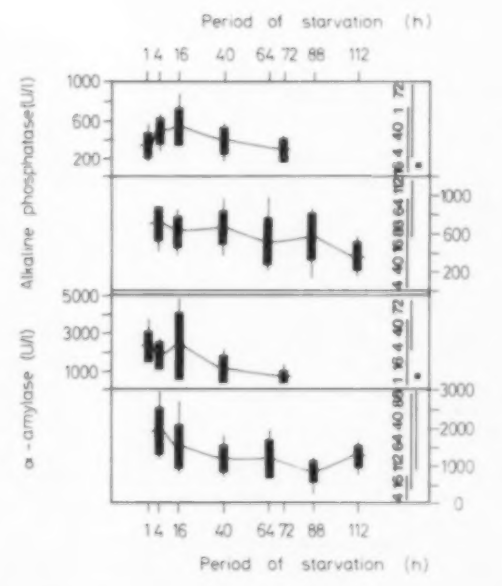

Fig. 4. Changes in plasma α-amylase and alkaline phosphatase activities during hunger in two groups of rainbow trout (I and II). Explanations, see Fig. 1.

in this experiment (the large trout of group I emptied their stomach after 16 hr, too). That is easily explained by the different feeding modi in the literature (force-fed to satiation) and in this paper (regularly fed with size adjusted ration), for which reason the slowly digesting large fish emptied their smaller ration in the same time that the small trout needed for their higher ration (Jobling *et al.*, 1977).

The asymptotic reduction of the gastric plus intestinal content corresponds to the results of Schade and Kausch (1978) with carp fry.

The increase of glucose and lipid level soon after feeding should be due to food resorption and transport via the blood. The glycemic changes correspond to the data of Bergot (1979a, b), who observed a glucose maximum at 6 hr after food ingestion. The consequent constancy of glucose and lipid, and the constant protein levels show that these trout did not get into a state of severe depletion, whereas in the course of prolonged starvation the glucose and protein concentration decreased (Shimma *et al.*, 1976a, b; Milne *et al.*, 1979; Kawatsu, 1974), and the lipid content increased partially (Bilinski and Gardner, 1968; Shimma *et al.*, 1976a).

During prolonged starvation the blood sodium and chloride concentrations of different fishes decreased (Love, 1970), which was explained by reduction of intracellular volume, compensated by an increase in extracellular volume in the form of water incorporation. That means, that the blood (deriving from the extracellular volume) was diluted, i.e. the sodium and chloride concentration of the plasma decreased. In contrast, in this short term experiment, the sodium and chloride content of the blood of hungry trout increased. Here, the explanation should be searched in the physiological processes of digestion. As in mammals, the hydrochloric acid secretion of fishes is stimulated by food ingestion. Consequently, whereas empty stomachs of rainbow and brown trout have pH values above 5, the pH decreases after food ingestion to 2–3 (Fish, 1960; Reichenbach-Klinke, 1969). In mammals it is shown that the chloride ions of this gastric acid derive from the blood, primarily. They are exchanged for hydroxide ions in the parietal cells and transported actively into the stomach. In the small intestine $NaHCO_3$ is secreted to adjust alkaline pH. The electrolytes are reabsorbed during ingestion and return to the blood (Gauer *et al.*, 1972). In this way the minimum concentrations of sodium and chloride in the trout blood after feeding should be due to the delivery of electrolytes for the gastric and intestinal secretion. The consequent increase of the sodium and chloride content is certainly caused by the reabsorption of these electrolytes. Only after prolonged starvation the electrolyte content of the blood should diminish again.

However, AP and α-amylase decreased in the course of food deprivation even in this short term experiment. Both enzymes take a share in the digestion and transport of food molecules (Boyer, 1960) and seem to decrease in case that they are not needed. Noda (1967) demonstrated that the AP activity of the intestine epithelium decreases already at the beginning of starvation.

With hematological studies in trout, the stated changes of blood concentrations demand a standardization of the time of blood withdrawal in relation to the cessation of feeding; 16 hr is considered a suitable standard time for further experiments.

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RELATIONSHIP BETWEEN THE INTRA-ERYTHROCYTE SODIUM COMPOSITION AND THE MEMBRANE LIPOPROTEIN COMPOSITION AMONG DIFFERENT MAMMAL SPECIES

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Abstract—1. Internal sodium and lipoprotein composition of RBCs of nine mammalian species are measured.

2. A significant correlation can be demonstrated between the erythrocyte mean sodium value of studied species and the membrane protein/lipid ratio ($r = 0.80$, $\alpha < 0.01$).

3. Erythrocyte internal sodium can be correlated with membrane-free cholesterol but not with the phospholipid fractions.

INTRODUCTION

Recent studies have shown that for man, modification of erythrocyte cationic flux could be seen in some diseases without any apparent link between them, such as arterial hypertension, familiar obesity or manic depressive psychosis (Loss *et al.*, 1981; Hesketh *et al.*, 1977; De Wise *et al.*, 1980).

Regarding arterial hypertension, Garay *et al.* (1980) have shown a genetic transfer of the disease by the dominant autosome mode, and by measuring the cotransport of lithium. Levy *et al.* (1982) have suggested that the anomaly of the membrane could be due to the organization of lipids and proteins of the membrane.

During this study, we determined intra-erythrocyte sodium level among different mammal species, as well as the concentrations of the different categories of erythrocyte-lipids, in order to see the eventual role of the lipids in ionic exchanges.

MATERIALS AND METHODS

Choice of the animals

The animals were chosen according to the lipid composition of their red cells, such as was reported by Van Dennen and De Gier (1974). They were as follows: 5 dogs, 5 cats, 5 goats, 5 rabbits, 5 calves, 3 sheep, 5 horses, 4 pigs.

The different values will be compared to the values found for man.

Assay of intra-erythrocyte sodium

Intra-erythrocyte sodium was in the newly sampled plasma and in the supernatant of a 5% trichloroacetic defecation (1:2 v/v) was assayed by flame emission spectrometry.

Sodium value is obtained by the formula:

$$Na_i = \frac{\text{Measured sodium} \times F - \text{Plasmatic sodium}}{\text{Hematocrit}} \times (1\text{-hematocrit})$$

Meaning of the different symbols: Na_i = Intra-erythrocyte sodium; Na_P = Plasmatic sodium; Na_M = Measured sodium; and F = Dilution factor.

The dilution factor was experimentally determined taking into consideration the hemoglobin concentration. It varied as a straight line when related to the hemoglobin concentration, and it corresponds to the internal cell volume.

Assay of plasma and erythrocyte lipids

The erythrocyte membranes of the different red cells were obtained according to Dodge *et al.* (1962). Membrane proteins were assayed according to Lowry *et al.* (1951). The lipids were extracted according to Folch *et al.* (1951), using chloroform/ethanol extracting mixture (2:1 v/v). Phospholipids were separated by silica gel thin-layer chromatography in a chloroform/methanol/water developing solvent (65:25:4 v/v/v). Neutral lipids were separated in a standard benzene/diethylether/acetic acid developing solvent (90:10:1 v/v/v).

The different categories of lipids were quantified according to Pré and Garnier (1973) by coloring the spots with amidoblack and 5% acetic acid. As the final volume of membrane suspension was equal to the volume of the red cells sample, the results could be explained in mg/10^{10} red cells.

RESULTS

Intra-erythrocyte sodium concentration among different mammal species

The values of intra-erythrocyte sodium among different mammal species are given in Table 1.

They agree with those previously published by Tucher and Eloi (1973) for sheep, Thiele *et al.* (1979) for calves, Katanic *et al.* (1976) for cows, Galter and Small (1973) for dogs, and Gattegno *et al.* (1974) for rabbits.

The different species used during this study enabled us to obtain red cells in which the intra-erythrocyte sodium concentration varied between 13 mEq/l of red cells for the pig, and 126 mEq/l of red cells for the dog.

Red cell membrane lipoprotein composition among the different species of studied mammals

The quantity of membrane proteins obtained from red cells of different mammal species varied from 2.72 $\pm$0.47 mg per 10^{10} red cells for the cat, up to 5.2 $\pm$0.46 per 10^{10} red cells for man.

Table 1. Lipoprotein composition and internal sodium among nine mammal species

Species	Sodium intra (mEq/l)	MCV (μ^3)	Proteins (mg/10^{10} RBC)	Lipids (mg/10^{10} RBC)	Proteins/lipids
Pig	10±4	68±5	4.01±0.25	3.58±0.12	1.12
Man	15±5	90±4	5.20±0.46	5.00±0.34	1.04
Rabbit	44±10	44±4	3.24±0.46	3.20±0.12	1.01
Goat	81±31	16±5	4.42±0.80	4.35±0.71	1.02
Horse	97±13	47±6	3.14±0.26	3.44±0.15	0.94
Calf	103±19	50±4	3.12±0.09	2.87±0.23	1.02
Sheep	105±10	37±7	3.27±0.52	3.53±0.33	0.93
Cat	119±9	55±7	2.72±0.47	3.57±0.08	0.76
Dog	126±7	64±6	4.11±1.16	5.29±0.34	0.77

The quantity of erythrocyte lipids varied in the same way as the proteins, but the protein/lipid ratio varied according to the species—between 0.76 for the cat and 1.04 for man.

Lipid composition of the different categories of red cells

The values of erythrocyte lipids reported in Table 2 are in good agreement with those given by Van Dennen and De Gier (1974). The selected species enabled us to obtain on the one hand red cells devoid of phosphatidylcholine, such as those obtained from calf, goat and sheep, and on the other hand red cells showing big variations in the different categories of phospholipids, particularly sphingomyelins which vary between 0.30 mg and 1.37 mg per 10^{10} red cells, phosphatidylethanolamines which vary between 0.63 and 0.97 mg per 10^{10} red cells, and phosphatidylserines which vary between 0.36 and 0.79 mg per 10^{10} red cells.

Statistical study of the relationship between the variations of intra-erythrocyte sodium and the modifications of the different categories of membrane lipids

Statistical studies showed that there was no correlation between the variations of erythrocyte corpuscular volume and the intra-erythrocyte sodium content. Goat red cells, which had a 16 μ^3 MCV, had 80 mEq of Na^+/l of red cells, whereas rabbit with a 44 μ^3 MCV had 44 mEq of Na^+/l of red cells and dog with a 64 μ^3 MCV had 126 mEq of Na^+/l of red cells.

Relationship between membrane protein/lipid ratio and intra-erythrocyte sodium concentration

As Fig. 1 shows, there is a highly significant correlation ($r = 0.80$, $\alpha < 0.01$) between the erythrocyte membrane protein/lipid ratio and the intra-erythrocyte sodium concentration. The higher the ratio, the lower is the erythrocyte sodium concentration. One can find pig and dog species at the two extremities of the straight line.

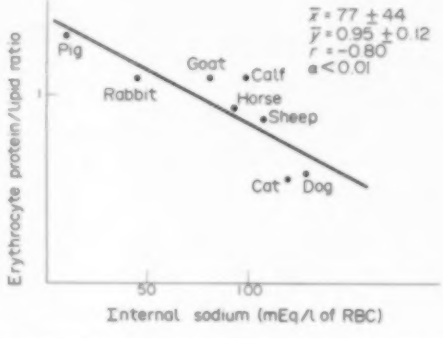

Fig. 1. Correlation between internal sodium and protein/lipid ratio among nine mammal species.

When results are explained in mg of lipid per 10^{10} red cells, there is no statistically significant relationship between the variations of intra-erythrocyte sodium and the values of the different phospholipid fractions, nor with the membrane cholesterol/phospholipid ratio. On the other hand, when results are explained in lipid mg/protein mg, one can find a statistically significant correlation with a 5% cut-off between the variations of membrane free cholesterol and variations of sodium ($r = 0.60$, $\alpha < 0.05$).

Table 2. Erythrocyte lipids in mg/mg of proteins among nine mammal species

Species	P.S.	P.C.	S.P.	P.E.	F.C.	F.C./P.L.
Cat	0.22±0.05	0.34±0.05	0.17±0.02	0.23±0.01	0.27±0.07	0.27
Pig	0.19±0.02	0.24±0.04	0.06±0.03	0.25±0.02	0.21±0.02	0.29
Sheep	0.20±0.02	0.00±0.00	0.37±0.04	0.24±0.02	0.25±0.04	0.30
Human	0.12±0.02	0.46±0.05	0.08±0.02	0.20±0.01	0.20±0.02	0.31
Calf	0.22±0.03	0.00±0.00	0.22±0.02	0.28±0.01	0.23±0.02	0.31
Horse	0.11±0.02	0.31±0.04	0.13±0.02	0.21±0.02	0.28±0.03	0.36
Dog	0.19±0.03	0.22±0.03	0.14±0.02	0.26±0.02	0.34±0.03	0.36
Rabbit	0.13±0.02	0.26±0.04	0.09±0.01	0.19±0.03	0.21±0.02	0.43
Goat	0.14±0.02	0.00±0.00	0.06±0.01	0.21±0.02	0.31±0.02	0.34
Correlation coefficient	0.34	0.42	0.46	0.57	0.60	0.02
α	N.S.	N.S.	N.S.	<0.10	<0.05	N.S.

P.S. = phosphatidylserine, P.C. = phosphatidylcholine, S.P. = sphingomyelin, P.E. = phosphatidylethanolamine, F.C. = free cholesterol, F.C./P.L. = free cholesterol/phospholipid ratio.

DISCUSSION

These study results show that, if taken separately, there is no significant correlation between the different membrane phospholipid components and the intra-erythrocyte sodium concentration, nor with the quantity of membrane proteins. On the other hand, the very significant correlation found between the protein/lipid ratio and the sodium concentration shows that the mechanism of regulation is of a lipoprotein kind.

Among a few mammal species, such as man, rabbit and pig, there is, in the red cell membrane, a sodium pump, inhibited by ouabain, which pumps against a strong concentration gradient (Rettori *et al.*, 1968; Viglíocco *et al.*, 1970). In other species, such as dog, cat, sheep and cow, membranes mostly lack this sodium pump (Tosteson and Hoffman, 1961; Israel *et al.*, 1972). In species with a sodium pump the intra-erythrocyte sodium concentration is therefore low, whereas it is high in species without a sodium pump.

Considering man, previous studies have shown that (Na^+K^+) ATPase activity and ($Mg^{2+}Ca^{2+}$) ATPase activity needs the presence of glycerophospholipids located in the internal face of the lipid double layer. Only phosphatidylserines are needed for (Na^+K^+) ATPase activity, whereas all phospholipids located on the internal face are needed for ($Mg^{2+}Ca^{2+}$) ATPase activity (Roclofsen and Van Deenen, 1973; Roclofsen and Schatzmann, 1977).

It is only by using free cholesterol in mg/mg of proteins as a parameter that one can find a statistically significant correlation with a 5% cut-off.

In human intact erythrocyte membranes, there is no cholesterol in the close area around (Na^+K^+) ATPase; it is therefore possible that the increase of erythrocyte membrane-free cholesterol might have an inhibitory effect on ATPase, and therefore might increase the intraerythrocyte sodium concentration.

This possible action of membrane-free cholesterol has been suggested by authors who have shown that by adding free cholesterol to erythrocyte membranes, either the sodium pump could be inhibited and sodium efflux slowed down, or this efflux could be elevated by increasing the affinity of the pump for internal sodium. Subjects showing hepatic diseases involving increased red cell free cholesterol and phosphatidyline (Owen and McIntire, 1978) have demonstrated a very significant correlation between the erythrocyte sodium and the membrane cholesterol/phospholipid ratio ($r = 0.66$, $\alpha < 0.01$).

The present study carried out using red cells of different mammal species therefore shows the important role of lipids, particularly free cholesterol, in the regulation of intraerythrocyte sodium concentration. In spite of numerous studies the distribution of free cholesterol inside the lipid double layer is not yet well defined. The results explained in this study suggest that besides its structural role, cholesterol can have a metabolic role for which its localization related to membrane enzymatic proteins would be more important than its absolute value.

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VENTILATION IN AWAKE RATS WITH PERMANENT ARTERIAL CATHETERS

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Abstract—1. Respiratory frequency (f), tidal volume (V_T) and minute ventilation ($\dot{V}_E$) were measured in restrained awake rats using a volumetric body plethysmograph excluding the head. An arterial catheter was then implanted and ventilatory measurements performed again two or three days later. Arterial blood gases and acid–base status were measured before entry in the plethysmograph (control values) and after, accompanying each ventilatory measurement.

2. The pattern of ventilation (V_T and $\dot{V}_E$) was significantly correlated with the respiratory status, particularly with Pa_{CO_2} ($P < 0.001$).

3. A ventilatory ($\dot{V}_E$, V_T, f) and respiratory (Pa_{O_2}, Pa_{CO_2}, pH) steady state was obtained from the fifteenth minute to the end of the observation period.

4. The present preparation is a stable experimental model providing respiratory and ventilatory data in the awake rat.

INTRODUCTION

Repetitive measurements of physiologic parameters in unanesthetized animal are often indispensable to observe and quantify the processes regulating major functions. Thus, chronic arterial or venous catheter implantation allows the repeated evaluation of respiratory and acid–base blood status in rodents (Popovic and Popovic, 1960). The normal range of blood gas pressures, Pa_{O_2} and Pa_{CO_2}, as well as the values of the acid–base parameters pH and $[HCO_3^-]_a$ in the rat (Brun-Pascaud *et al.*, 1982) and the guinea pig (Pocidalo *et al.*, 1983), have been recently established under such conditions in the laboratory. Ventilatory pattern, frequency and tidal volume, can also be measured in unanesthetized animals using the plethysmographic technique. In the last few years, several authors have presented results concerning the ventilatory pattern of unanesthetized small laboratory animals using a barometric (Lai *et al.*, 1978; Nattie, 1977; Olson and Dempsey, 1978; Pappenheimer, 1977) or volumetric (Coggins *et al.*, 1981) method. Few simultaneous and repetitive measurements of both blood respiratory status and ventilatory pattern have been performed (Favier and Lacaisse, 1978) but no work has attempted to identify the conditions required for obtaining a reproducible steady state. The aim of the present study was to search for such conditions in the rat. On the one hand, ventilation was measured with a volumetric plethysmograph excluding the head of the animal and consequently avoiding the rebreathing encountered in whole-body plethysmographs. On the other hand, pH, Pa_{O_2}, Pa_{CO_2} and $[HCO_3^-]_a$ were determined after implantation of an intraaortic arterial catheter. It was thus possible to compare ventilatory patterns before and after catheter implantation.

MATERIALS AND METHODS

Animal preparation

The study was carried out in six adult male Sprague–Dawley rats weighing about 300 g. After ventilation was measured in intact rats, they were anesthetized and a catheter was inserted into the carotid artery. The cannula was tunneled subcutaneously and brought out at the back of the neck. Before sampling a long catheter was connected to the one already in place (Fig. 1a).

Plethysmograph

Ventilation was measured following the method of Coggins *et al.* (1981) modified by the fact that we removed the piece restraining the head of the rat. The plethysmograph is reproduced in Fig. 1(b) (made by Battelle, Centre for Toxicology and Biosciences, Geneva, Switzerland). The 0.4 l.

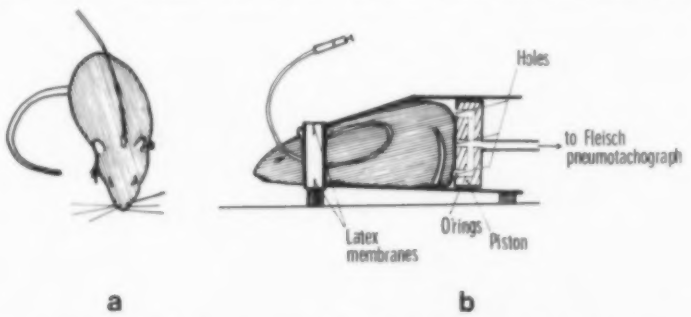

Fig. 1. (a) Catheter inserted into the carotid artery. (b) Catheterized rat in the plethysmograph.

(when adjusted for a rat) plethysmograph consists of a 20-cm cylindroconic tube with 7.6 cm largest diameter and a piston. The head of the rat emerges through an opening at the end of the conic part (dia 4.1 cm). The catheter is looped inside the plethysmograph and brought out with the head of the rat. Thus, a blood sample can be withdrawn immediately following respiratory determinations. Four latex rings are fitted around the rat's neck and the piston is fitted with rubber O-rings and sealed. The rats often enter the plethysmograph voluntarily and the piston is rapidly pushed ahead. Holes in the body of the piston, in communication with ambient air through the axis of the piston, transmit airflow due to the respiration of the rat to a Fleisch pneumotachograph (size 4.0) which creates a differential pressure proportional to the airflow. This alternating differential pressure is converted into electrical signals by means of a symmetrical differential capacitance-type pressure transducer (Statham PM 197c). These signals are amplified and integrated to give volume signals. The volume change caused by each breath is calibrated by injection of known air volumes into the Fleisch head from calibrated syringes.

Protocal

Two series of measurements were made for each rat: (1) before arterial catheter implantation, (2) at least 48 hr after implantation. Each time, ventilation was measured for one hour. As soon as the rat was quiet following entry in the plethysmograph (after 10 min maximum) flow and tidal volume were recorded (time point 0-min) on a graphic recorder (Hewlett-Packard 7702B) at 5 mm/sec. Measurements were repeated at 15-min intervals over one hour. In the second series measurements, arterial pressure was recorded and 0.4 ml of blood was withdrawn for arterial blood gases and acid–base status determination before the rat entered the plethysmograph. When the rat was inside the plethysmograph arterial pressure and ventilation were recorded, followed by withdrawal of blood. The arterial catheter was connected to a Statham P23 pressure transducer, enabling arterial pressure and cardiac rate to be recorded. Pa_{O_2} and Pa_{CO_2} were measured at 37.5°C using a Combi-Analysator (Eischweiler and Co, Kiel) as previously described (Blayo and Pocidalo, 1963) and pH was measured at 37.5°C with calibrated electrodes (Type E 5021, Radiometer, Copenhagen). Plasma bicarbonate concentration $[HCO_3^-]_a$ was calculated with the Henderson–Hasselbalch equation. Rectal temperature was measured at the beginning and the end of the series 2 measurements.

Statistical analysis

Between-time variations were studied by analysis of variance. Global time effect significance enabled the differences to be localized by calculating the least significant difference (taking, as the variance, the variance of the interaction period × rat) and comparing to it each of the differences between two time points. Ventilation values before and after catheter implantation were also compared by analysis of variance. Values were reported as means ± SD. We assumed that $P < 0.05$ was significant. Linear regressions were performed to study relations between two variables.

RESULTS

Ventilation in intact rats

Means of respiratory frequency (f), tidal volume (V_T) and minute ventilation ($\dot{V}_E$) in intact normal rats after 0, 15, 30, 45 and 60 min past in the plethysmograph are presented in Table 1. For V_T and $\dot{V}_E$ values, there was no significant difference between time points. For f, the 0-min time point was significantly higher than each of the 15, 30, 45 and 60-min points which did not differ between themselves. In other words, values found for f appeared stable from 15-min on. V_T was stable immediately while $\dot{V}_E$ seemed to follow the same trend as f although this was not statistically significant. Excluding the 0-min time point, the means ± SD for f, V_T and $\dot{V}_E$ were respectively $f = 116 \pm 21$ breaths/min (bpm), $V_T = 0.56 \pm 0.10$ ml/100 g body wt, $\dot{V}_E = 64 \pm 16$ ml/min/100 g body wt.

Ventilation after catheter insertion

Results obtained at the same observation times as above are shown in Table 2. For f and $\dot{V}_E$, the 0-min time point was significantly higher than the other time points which did not differ between themselves. Results followed the same trend as before catheter implantation. Thus, excluding the point 0, ventilatory status remained stable for the whole observation period from the fifteenth minute on. Means ± SD were $f = 104 \pm 12$ bpm, $V_T = 0.49 \pm 0.08$ ml/100 g body wt and $\dot{V}_E = 50 \pm 8$ ml/min/100 g body wt. Means of f, V_T and $\dot{V}_E$ were slightly lower after placement of the catheter

Table 1. Time course of ventilatory parameters in six awake normal rats restrained for up to 1 hr in a volumetric body plethysmograph

	Time in the plethysmograph (min)					Two-way
	0	15	30	45	60	anova
Frequency/min	153***(4)	120	123	115	108	P<0.01
	±16	±15	±30	±20	±18	
V_T, ml per 100 g	0.57	0.58	0.56	0.54	0.54	NS
	±0.17	±0.14	±0.10	±0.10	±0.09	
$\dot{V}_E$, ml.min^{-1} per 100 g	88	68	69	62	58	NS
	±32	±14	±23	±13	±11	

Values are means ± SD. Anova: analysis of variance. Time point 0-min is the time of the first measurement. Significance: ***for $P < 0.001$ using the Student t-test to compare the time points means, with, as the variance, the variance of the interaction period × rat; (4): as compared to each of the four times 15, 30, 45 and 60-min. A time point mean with no indicated significance or not concerned with a digit between parenthesis is not significantly different from the others. Dotted lines border group of means for all variables; the mean on the four times for each variable is given in the text.

Table 2. Time course of ventilatory parameters, blood gases and acid–base status in the same six rats as in Table 1 under the same conditions except for catheter implantation

		Time spent in the plethysmograph (min)					Two-way
	Control	0	15	30	45	60	anova
Frequency/min		$145^{***(4)}$ ±22	110 ±11	106 ±19	100 ±10	102 ±6	P<0.001
V_T, ml per 100 g		0.54 ±0.13	0.47 ±0.06	0.47 ±0.08	0.52 ±0.07	0.49 ±0.10	NS
$\dot{V}_E$, $ml.min^{-1}$ per 100 g		$78^{*(4)}$ ±18	51 ±8	48 ±3	52 ±10	50 ±10	P<0.001
Pa_{O2}, Torr	94 ±5	97 ±5	94 ±9	96 ±6	97 ±5	95 ±6	NS
Pa_{CO2}, Torr	$35^{***(1)}$, $^{*(4)}$ ±2	31 ±3	33 ±3	33 ±3	32 ±3	33 ±2	NS
pH	7.45 ±0.02	7.44 ±0.02	7.44 ±0.01	7.44 ±0.01	7.45 ±0.02	7.45 ±0.01	NS
$[HCO_3^-]_a$, $mmol.l^{-1}$	$24.6^{***(3)}$, $^{**(2)}$ ±1.1	$21.4^{*(2)}$ ±2.0	22.2 ±2.2	22.3 ±1.8	22.7 ±1.5	23.0 ±1.2	P<0.05

In addition to the legend of Table 1: $^*P < 0.05$, $^{**}P < 0.01$ as compared to (1) the time 0-min, (2) each of the two times 45 and 60-min, (3) each of the three times 0, 15 and 30-min.

than those obtained previously. A three-way analysis of variance indicates a significant mean difference for f and V_T; however the intensity of this change between catheterized and uncatheterized animals was variable from on rat to the other (interaction $P < 0.05$ for f, $P < 0.001$ for V_T). Such a rat-dependent variation for f and V_T in catheterized rats resulted in no significant difference in $\dot{V}_E$.

Blood gases and acid–base equilibrium (*Table* 2)

Pa_{O_2}, Pa_{CO_2} and pH remained stable throughout the observation period. At the time point 0, a trend to a metabolic process of acidotic type (pHa = 7.44 ± 0.02, Pa_{CO_2} = 31 ± 3 torr, $[HCO_3^-]_a$ = 21.4 ± 2 mmol/l) appeared when these values were compared with starting values i.e. before the rat was placed in the plethysmograph (pHa = 7.45 ± 0.02, Pa_{CO_2} = 35 ± 2 torr, $[HCO_3^-]_a$ = 24.6 ± 1.1 mmol/l). Excluding the 0-min time point, we found again the same stationary state as for ventilatory data: Pa_{O_2}, Pa_{CO_2}, pH and $[HCO_3^-]_a$ did not vary between 15 and 60 min Means ± SD where Pa_{O_2} = 95.4 ± 6.3 torr, Pa_{CO_2} = 32.6 ± 2.5 torr, pH = 7.4 ± 0.01, $[HCO_3^-]_a$ = 22.6 ± 1.6 mmol/l.

Rectal temperature before and after the period in the plethysmograph was 37.6 ± 0.1°C (mean ± SD) and 37.5 ± 0.1°C, respectively.

Relation between Pa_{CO_2} *and ventilatory status*

Figure 2 demonstrates the relationship between tidal volume and Pa_{CO_2}. The negative correlation ($r = -0.65$) was very significant ($P < 0.001$). The points corresponding to time 0 were not included, i.e. only times defining the respiratory and ventilatory stationary state, were taken into account.

A negative correlation ($r = -0.50$) could also be shown between Pa_{CO_2} and $\dot{V}_E$ ($P < 0.01$).

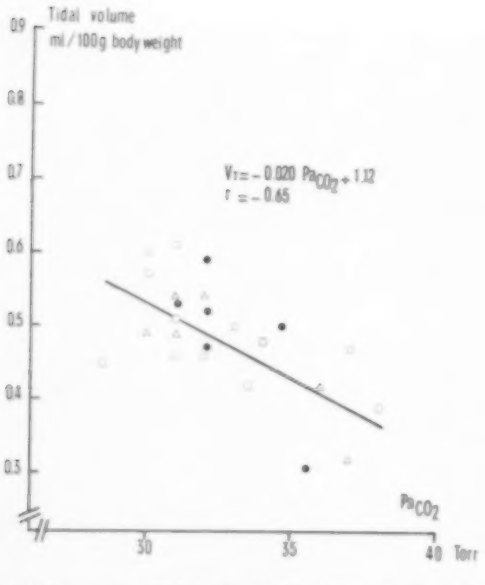

Fig. 2. Regression of tidal volume (V_T) on Pa_{CO_2} in six awake and catheterized rats restrained for up to 1 hr in the plethysmograph. Measurements at different times: ○ 15-min; △ 30-min; □ 45-min; ● 60-min.

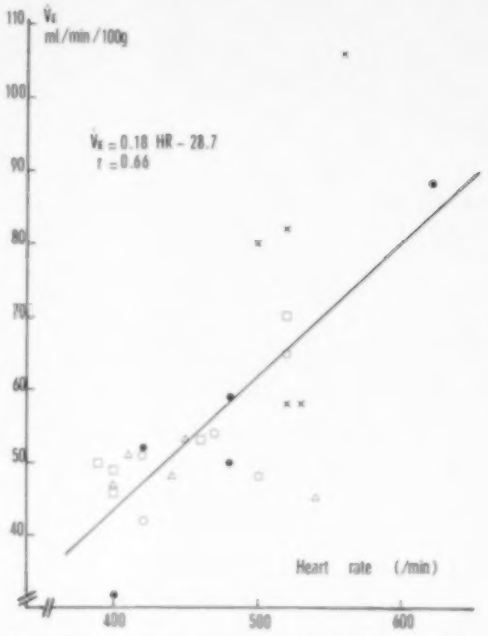

Fig. 3. Regression of minute ventilation ($\dot{V}_E$) on cardiac frequency. Conditions and symbols are the same as in Fig. 2; ×, 0-min (just after entry in the plethysmograph).

DISCUSSION

The present work validates an experimental model allowing simultaneous measurement of respiratory and ventilatory parameters, in the awake, chronically catheterized rat. The principal quality of this model is to provide steady conditions rather rapidly in spite of restraint (vascular catheter and body enclosure).

Table 3 shows ventilation values reported by different authors in awake rats. In their studies of ventilation these authors used a barometric or a volumetric whole-body plethysmograph. In practice, two different techniques can be opposed depending upon the dimension of the chamber. An exiguous chamber limits the activity of the rat, and owing to forced immobilization, permits the obtention of an adequate recording of the ventilation much faster and in a less aleatory way than when the rat can move freely inside the chamber. The technique used in the present study restrained the rat in a small volume chamber. The head of the animal was excluded outside the plethysmograph, thus avoiding the rebreathing that appears in a large chamber. A similar disposition had already been used in rat, twenty years ago, by Gottlieb and Jagodzinski but they had not given any ventilatory values.

Restraint explains certainly the higher values of f and $\dot{V}_E$ at time 0, i.e. when the rat has just entered the plethysmograph. Heart rate measurement was possible in the rats fitted with an aortic catheter. There is a good relationship between cardiac frequency and ventilatory level [$P < 0.001$ for $\dot{V}_E$ (Fig. 3) and $P < 0.01$ for f] indicating that hyperemotivity triggers an abnormally high ventilatory level, perhaps through a catecholamine release, at the same time as it influences the heart rate. But this situation is only transient because the frequency and pulmonary venti-

Table 3. Ventilatory blood gases and acid–base status measurements in unanesthetized rats weighing more than 200 g

Authors	Method	n	b.w. g	f b.p.m.	V_T ml/100g b.w.	$\dot{V}_E$ ml/min/100 g b.w.
BLUME and ZÖLLNER (1936,1943)	1	14	229	134	1.09	147
LEONG and al. (1964)	2 R	10	211.3±6.7	110.2±22.5	0.69	76
		10	298.5±7.6	121.4±7.7	0.62	75
BARTLETT and TENNEY (1970)	3 U	16	300-550	84.9±16.6	0.67	56.6±11.2
NATTIE (1977)	3 U	8	249.0±6.0	119.0±6.0	0.52	61
PAPPENHEIMER (1977)	3△U	5		83.0±3.2*(97)		47.0±2.8*(77)
FAVIER et LACAISSE (1978)	5□R	5	467	79±6*	0.78	60
LAI and al. (1978)	4×R	8	284±19	141±9	0.689±0.018	97.1±7.5
OLSON and DEMPSEY (1978)	3□U	67	303±12*	95±5*	0.83	78±3*
LAI and al (1981)	3×U	28	300	113.4±16.4	0.53±0.09	59.4±12.6
MASKREY and al (1981)	3 U	10	200-250	104±10*	0.479±0.024*	49.8±5.6
Present study (1982)	5 R	6	296±11	116±21	0.56±0.10	64±16
	5+R	6	294±17	104±12	0.49±0.08	50±8

Values are means ± SD, except when *: 2SE; *n*, number of rats; b.w., body weight; b.p.m., breaths/min; ( ), number of observations. Method 1, spirograph, head mask; method 2, respirographic technique with rat in a holder; method 3, 6.5–7-1. Barometric whole-body plethysmograph; method 4, approximately 2-1. Barometric whole-body plethysmograph; method 5, 0.5 to 2-1. Volumetric whole-body plethysmograph excluding the head; R, restrained rat; U, unrestrained; △, five implanted electrodes; ×, catheter inserted into the caudal artery; □, catheter inserted into the abdominal aorta; +, catheter inserted into the carotid artery.

lation decrease rapidly while the cardiac frequency returns to normal. After fifteen minutes, a steady state is established and analysis of variance reveals no significant modification at the subsequent times of the study, i.e. 15, 30, 45 and 60 min. The question remains whether, under these conditions, respiratory rate and ventilation are those of the normal resting rat. Taking into account the diversity of techniques used in previous studies it is difficult to compare our results with those in the literature. Favier and Lacaisse (1978) worked under experimental conditions similar to ours since ventilation measurements were made in previously catheterized rats. As their rats have a lower f and a greater V_T than ours, results are rather different. However these differences are possibly due to the age and weight of the rats that are inferior in our experiments.

Restraining the rat in its chamber has the disadvantage we just mentioned but involves another risk, that of a leak around the collar which would diminish the tidal volume and hence the minute ventilation. The existence of a leak at the level of the neck can be excluded since in each experiment the correct position of the rat was controlled. Besides these verifications, the absence of leak is confirmed simply by the good reproducibility of the measurements. Indeed, a leak around the neck resulting from a bad posture of the rat is not likely to be of constant magnitude. In addition, values presented in this study are comparable with those from another recent work where rats were left unrestrained inside a 6.5 l. plethysmographic chamber (Maskrey *et al.*, 1981).

Our protocol revealed differences in ventilation before and after aortic catheter implantation. This procedure, when chronic, alters the nutritional behaviour of the rat. The diminution of the food intake reduces the metabolism, including CO_2 production, and the expected ventilatory adaptation is indeed a reduction of respiratory frequency and tidal volume. This underlines the particular character of the present model of awake rat with an implanted catheter.

Blood gases and acid–base equilibrium were determined just following ventilation measurements. Validation of ventilatory measurements can be founded upon the significant correlations between ventilatory pattern (V_T and $\dot{V}_E$) and the respiratory state as judged by the level of Pa_{CO_2}. Pa_{O_2} remained stable throughout the observation. Acid–base values were appreciably modified after entry of the animals in the plethysmograph. The decrease of the bicarbonate concentration was highly significant, unlike that of pH. Such an event can be interpreted as a metabolic one due to the probable increase of catecholamines also responsible for the acceleration of the heart rate. As early as 15 min, the analysis of variance showed a simultaneous ventilatory (f, V_T, $\dot{V}_E$) and respiratory (Pa_{O_2}, Pa_{CO_2}, pH) steady state lasting until the end of the observation, i.e. 60 min, while plasma bicarbonate concentration increased again.

In conclusion, despite the inherent peculiarities mentioned above, the present preparation provides an experimental model that allows the rapid production of reproducible respiratory and ventilatory data in the awake rat.

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THE COST OF WALKING IN CRABS: AERIAL AND AQUATIC OXYGEN CONSUMPTION DURING ACTIVITY OF TWO SPECIES OF INTERTIDAL CRAB

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Abstract—1. The amphibious inertidal crab *Pachygrapsus marmoratus* consumed oxygen at almost equal rates in air and sea water during activity and when inactive. The low shore *Carcinus mediterraneus* when active or inactive respired at between 25 and 44% of the aquatic rate in air.

2. Oxygen consumption in sea water increased linearly with walking speed for both species. After a 10 min period of exercise oxygen debts of between 40 and 100% of the net oxygen consumption during exercise were accumulated and repayed within 20 min.

3. Costs of locomotion in sea water were temperature independent and similar to values for walking invertebrates and vertebrates.

4. The circulatory adjustment during exercise included lowering of arterial and venous pO_2, maintenance of arterial to venous difference, and an increase in stroke volume.

INTRODUCTION

Around the rocky coasts of Europe a common crab that can be found from the lower to the upper shore is *Pachygrapsus marmoratus* (Fabricius). The crab can be seen feeding in air and sea water and is a true semi-terrestrial species (Ingle, 1980). In contrast *Carcinus mediterraneus* Czerniavsky is found from mid to low shore and in sea water lagoons and was not observed to be active in air on the shore. The present paper investigates whether these differences in activity and tidal zonation are reflected in the relative capabilities of the crabs to respire in sea water and air as has been found in recent investigations with intertidal gastropods (Houlihan and Innes, 1982 a, b).

The rates of oxygen consumption of inactive animals in sea water and air have been measured at known periods since handling as inactive rates of oxygen consumption of decapods have been found to be very variable (Ansell 1973; Aldrich and McMullan, 1979; Burnett and Bridges, 1981).

Oxygen consumption during activity has been investigated by two methods. Firstly animals were stimulated to activity in small respirometers in air and sea water. Together with measurements of oxygen consumption of inactive animals this allows an estimate of the ratio of the maximal to the resting oxygen uptake, designated by Drabkin (1950) as the "index of expansibility of metabolism". It would be expected that the aerobic expansibility in air would be greater for *Pachygrapsus* than for *Carcinus* correlating with their activity and zonation.

Another measure of oxygen consumption during activity is the relationship between walking speed and oxygen consumption. Such measurements have been made for air-breathing crabs (Herreid *et al.*, 1979; Herreid, 1981); and these studies have found a linear relationship between oxygen consumption and walking speed. This has also been found for many vertebrates (Taylor, 1982). After periods of exercise crustaceans are reported to show long periods of repayment of an oxygen debt (McMahon *et al.*, 1979; Wood and Randall, 1981a). This paper reports on oxygen debts in sea water after a defined period of exercise.

An important component in the differences in behaviour between *Pachygrapsus marmoratus* and *Carcinus mediterraneus* is the observable difference in activity in air. In an attempt to quantify this difference the two species have been exercised in air at environmental temperatures and the distance walked, duration of walking and speed recorded. The performances in air have been compared with those in sea water. Rather than use the burst speeds investigated by Burke (1979), which are largely fuelled by anaerobic metabolism, fast prolonged speeds which are principally aerobic in small *Carcinus maenas* (Houlihan *et al.*, unpublished) have been used.

Previous reports on blood oxygen levels in crabs have pointed out that there is a drop in blood oxygen content during or immediately after activity (Johansen *et al.*, 1970; Mangum and Weiland, 1975; McMahon *et al.*, 1979; Wood and Randall, 1981a; Booth *et al.*, 1982). Heart rate has been reported to decrease (Herreid *et al.*, 1979) and increase (Wood and Randall 1981a) during exercise in crabs; one of the major circulatory adjustment during exercises seems to be an increase in stroke volume (McMahon *et al.*, 1979; Wood and Randall, 1981b; Booth *et al.*, 1982). The present paper reports on the circulatory changes after severe exercise in sea water.

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MATERIALS AND METHODS

Animals

Specimens of *Pachygrapsus marmoratus* were collected from the intertidal zone; *Carcinus mediterraneus* was collected from marine lagoons and was identified using the criteria of Rice and Ingle (1975). The experiments were carried out at the Laboratoire Arago, Baryuls-sur-Mer, France in July and August, 1980 and 1981. The sea water temperatures ranged from 18 to 25°C on the shore and the air temperatures varied from 20 to 30°C (Houlihan and Innes, 1982a). Salinity was 37‰. The crabs were kept in running sea water at 20°C without food and experiments performed within two to four days of collection. Animals were brought from the aquarium to the laboratory the night before measurement and were only handled as they were placed in the respirometers. The animals were kept under a natural light regime. For experiments at 30°C the animals were brought to that temperature over several hours in sea water and they experienced at least 12 hr at 30°C before experiments were performed. Both male and female crabs were used.

Aquatic oxygen consumption

Oxygen consumption of individual animals was measured in either small respirometer bottles or in a circular exercise respirometer. The respirometer bottles were fitted with a magnetic flea which was either separated from the animal with a plastic shield and only used for stirring the water or was in contact with the animal and used to illicit activity. Individuals were placed in the respirometers and aerated sea water was flushed through for 20 min. Water samples were taken from the bottles which were then sealed. Experiments were carried out either on animals seen to be inactive during the course of the measurement or on animals induced to be maximally active by rotating the bottle and using the magnetic flea. At the end of the experiments, which lasted for about 5 min, the water in the respirometer was thoroughly stirred with the flea, samples drawn off with a syringe and their oxygen content determined. Experiments were also carried out in which the animals were placed in the respirometer bottles with flowing sea water and kept overnight in the dark. Oxygen consumption was measured the next morning without disturbing the animals. Such animals are described as settled in the text.

Measurements of walking speed and oxygen consumption in sea water were carried out in the apparatus shown in Fig. 1. After the crab was placed in the respirometer (1.45 l vol) the lid was sealed and aerated sea water flushed through for several min. Two samples of sea water were taken from inside the respirometer which was then sealed and the crab made to walk around the tank by gently touching the animal with a magnetic flea manipulated by a magnet outside the tank. The water currents produced by the animal's movements and by the magnetic flea were minimal. This was shown by placing small polystyrene balls in the respirometer and noting that they hardly moved during an experiment. The time for each lap of the respirometer was recorded and kept as constant as possible during the course of the experiment. The mean standard error for lap times within an experiment was ±1.1 sec. After a period of from 5 to 10 min the water in the respirometer was thoroughly stirred with the magnetic flea and two samples of sea water were taken and their oxygen content determined. In both types of respirometer the oxygen consumption was calculated from the decline in pO_2 of the water, the experimentally determined relationship between pO_2 and oxygen solubility (determined with the Winkler technique, Strickland and Parsons, 1972), the volume of water in the respirometer, and the duration of the experiment. A respirometer without an animal was used as a control with each experiment. In both respirometers the pO_2 of the closed system was never allowed to fall below 90% of air saturation and the two respirometers gave identical resting rates with the same animal. In order to determine oxygen debts, individual animals were exercised in a large open tank and the oxygen consumption subsequently measured over 2 min periods in the small respirometers with aerated sea water being flushed through between determinations. The animals were inactive in the small respirometers.

Aerial oxygen consumption

Aerial oxygen consumption of individual animals was measured using a constant pressure differential respirometer described by Houlihan and Sell (1982) with 20% KOH on filter paper acting as a CO_2 absorber (Fig. 1). Individual animals were placed in the respirometer bottles which were dampened with sea water and contained a large magnetic flea. After 20 min in a constant temperature bath, the respirometer was sealed and measurements of oxygen consumption made every 2 to 3 min. Measurements were made either on animals seen to be inactive over a 20–30 min period or on animals stimulated to maximum activity with a rotating magnetic flea driven by a submersible magnetic stirrer (Fig. 1). Activity usually continued for 5 min. All aerial and aquatic rates were converted to STPD.

Heart rate and blood samples

A dental drill was used to pierce 1 mm diameter holes through the carapace on either side of the heart. Insulated wires were anchored into the holes with cyanoacrylate glue.

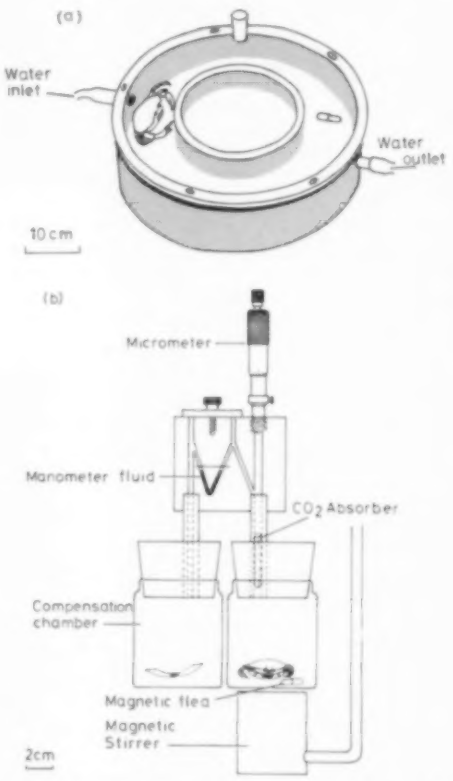

Fig. 1. (a) Diagram of apparatus used to measure walking speed and oxygen consumption in sea water. Individual animals were induced to walk at constant speed with the magnetic flea and the decline in oxygen content in the closed volume of water measured after 5–10 min. (b) Apparatus used to measure oxygen consumption in air. Movements of the manometer fluid due to oxygen uptake were compensated for with the micrometer. Measurements were made on animals seen to be inactive or on animals induced to be active by the magnetic flea driven by the submersible stirrer.

Impedence charges were measured with a Biocom impedence converter and recorded on a Washington pen recorder. The wires did not interfere with locomotion; some measurements were made with a non-invasive technique (Depledge, 1978) which gave results which did not differ from those obtained from wires inserted in the body near the heart.

Separate animals had a hole drilled over the heart, care being taken not to pierce the epithelium. Blood samples (0.2 ml) were drawn anaerobically into ice-cold syringes from the pericardial sampling site (arterial blood) and from the arthrodial membrane over the sinus at the base of the walking leg (venous blood). Blood was analysed within 2 min of sampling.

Blood samples were analysed for pO_2 and pH using radiometer equipment. The pH electrode was calibrated with radiometer precision buffers. Total O_2 content (C_{O_2}) was determined on 80 μl samples with a Lex-O_2-Con TL as described by McMahon *et al.* (1979). Water sample pO_2 was also determined with a radiometer electrode.

Exercise procedure

Crabs were exercised in a 29 cm dia circular tank with a hard, ridged plastic floor. They ran in response to being chased with a ruler. The time for each lap of the tank was recorded until the animal was judged to be exhausted, i.e. when it could no longer right itself. The direction in which the animal moved was alternated at least every 3 min or if the animal spontaneously changed direction. The exercise tank was held in a water bath at 30°C for sea water or air experiments at that temperature; otherwise experiments were carried out at the room temperature of 20°C. Animals were only exercised once and had a complete complement of legs and claws.

At the end of each experiment on each animal they were weighed in air, killed, and then dried at 70°C for 2 days or until they reached constant weight. With the experiments in air and water using the small respirometer a weight range of animals was used. For the speed vs oxygen consumption determinations, and the exercise experiments, animals in a small dry weight range were used. The mean dry weight of the *Pachygrapsus* used for these experiments was 4.37 ± 0.24 and 5.76 ± 0.27 g for *Carcinus*. Five gram dry weight is used as a standard sized animal for many of the calculations. The oxygen consumption of each experimental animal in the walking speed experiments was transformed to a standard sized animal using the scaling equation described previously (Houlihan and Innes, 1982b). A weight exponent of 0.65 was used for the scaling. Using other exponents from 0.55 to an exponent of 0.75 did not significantly alter the result. Relationships between dry weight and live weight were determined for both species by regression analysis; the whole live weight equivalent to 5 g dry wt is 15.2 g and 14.5 g for *Carcinus mediterraneus* and *Pachygrapsus marmoratus* respectively. The whole live weights have been used for some of the cost of transport calculations. Results were subjected to regression and covariance analysis and Student's *t*-test as described by Snedecor and Cochran (1972). The results of regression analysis using data transformed by logarithms to the base 10 are shown in their logarithmic form. Means ($\pm$SE) and the 5% level of significance have been used throughout.

RESULTS

Oxygen consumption in sea water: Carcinus mediterraneus

Oxygen consumption in sea water, of inactive animals measured over 20 min after a short period in the respirometer, was related to the animal's weight. Regression analysis of dry weight and oxygen consumption gives a significant correlation and the analysis account for 76% of the variation in oxygen consumption (Table 1). When the animals were stimulated to activity in the enclosed space of a small respirometer their oxygen consumption significantly increased above the level of consumption of inactive animals at both 20 and 30°C. (Fig. 2). The slopes of the oxygen consumption/weight regression analyses for the active animals are not significantly different from those of the inactive animals. Thus across the weight range of the animals used the aerobic expansibility is around 1.5-fold. When the animals were given an overnight settlement period there was a 3-fold decline in their inactive rates of oxygen consumption.

The increase in temperature significantly increased O_2 consumption of both the inactive and active animals ($P < 0.001$ in both cases) and gives Q_{10} values of around 1.4 for both inactive and active animals. Generally changes in temperature and activity did not significantly alter the slopes of the regression analysis shown in Table 1.

The animals could be induced to walk at a constant speed in the circular respirometer. The animals initially darted forward and then stopped but within a few circuits settled into a more constant pace. The oxygen consumption over a 5 min period increased as the walking speed increased. (Fig. 3). A narrow weight range was used for these experiments but it was found that converting the values to those for a standard 5 g dry wt animal as described in the Materials and Methods, significantly improved the correlation between walking speed and oxygen consumption. The data was subjected to regression analysis using a linear

Table 1. Regression analyses of dry weight (X, g) and O_2 consumption (Y, ml O_2/hr) for *Carcinus mediterraneus* and *Pachygrapsus marmoratus* in sea water

Species	Condition	Regression equation	r
Carcinus mediterraneus	20°C Settled	$\log Y = 0.593 \log X - 0.517$	0.871
	Inactive	$\log Y = 0.815 \log X - 0.185$	0.914
	Active	$\log Y = 0.720 \log X + 0.030$	0.925
	30°C Inactive	$\log Y = 0.599 \log X + 0.094$	0.806
	Active	$\log Y = 0.597 \log X + 0.252$	0.920
Pachygrapsus marmoratus	20°C Inactive	$\log Y = 0.654 \log X - 0.267$	0.934
	30°C Inactive	$\log Y = 0.410 \log X + 0.059$	0.800

Settled animals were left undisturbed in the respirometers for 12 hr; inactive and active animals were given 1 hr in the respirometers before the measurements were made; active animals were stimulated to activity in the respirometers. Each regression analysis was calculated from at least 15 determinations. r, correlation coefficient.

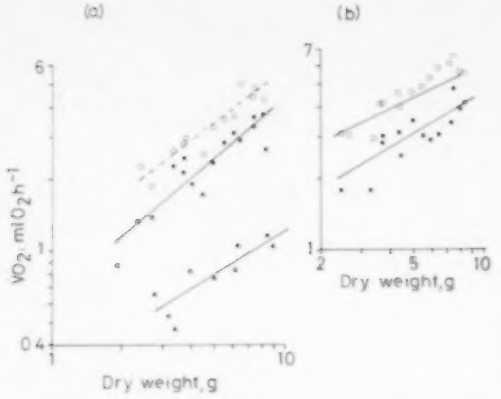

Fig. 2. Relationships between oxygen uptake in sea water and body weight for *Carcinus mediterraneus* at rest (●) and during activity induced in a small respirometer (○). Measurements from settled animals are also shown (▲). The lines are from the regression analysis shown in Table 1. (a) 20°C, (b) 30°C.

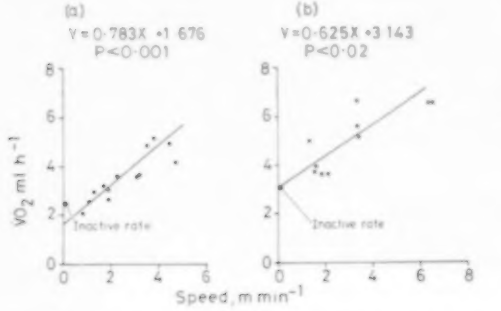

Fig. 3. Rate of oxygen consumption in sea water as a function of walking speed for *Carcinus mediterraneus*. Each point is from a single animal and has been calculated for a 5 g dry wt animal. The lines are from the regression analysis shown where Y is oxygen consumption (ml O_2/animal/hr) and X is speed (m/min). The rates of oxygen consumption from inactive animals are not included in the regression and are taken from Table 1. (a) 20°C, (b) 30°C.

model and gave significant correlation coefficients (Fig. 3). A semilogarithmic model has been used for studies on swimming animals (Brett, 1972; Quetin *et al.*, 1978) and this model was also used on the data. With both types of model there was a significant correlation between walking speed and oxygen consumption. On the basis of the coefficient of determination (r^2) there is no clear superiority in either model and the arithmetic model is used throughout. The intercept of the regression is lower than the rate of oxygen consumption for an inactive 5 g animal at 20°C and slightly above the rate at 30°C (Fig. 3). These inactive rates are within the 95% confidence limits of the intercepts and are not considered to be significantly different from them. The maximum increase in oxygen consumption was about 2.2-fold; this was a greater increase than that obtained from stimulating activity in the small respirometers. An increase in temperature increases the elevation of the speed vs consumption line ($P < 0.05$) but does not significantly alter the slope.

In order to determine the oxygen debt after severe exercise animals were exercised at a mean speed of 4.8 ± 0.44 m/min for 9.04 min, i.e. at about their maximum speed and duration (Table 3). Oxygen consumption for a standard animal at this level of walking was calculated from the speed vs oxygen consumption graph shown in Fig. 3. After the exercise oxygen consumption declined to pre-exercise levels within 20 min (Fig. 4). The amount of the oxygen debt was calculated from the shaded area shown in the figure. It amounts to 0.242 ml O_2.

The net increase in oxygen consumption associated with walking can be calculated as 0.522 ml O_2 and the oxygen debt is therefore 46% of the extra oxygen consumed during walking.

Oxygen consumption in sea water: Pachygrapsus marmoratus

Oxygen consumption of inactive animals was related to weight. Regression analysis of dry weight and oxygen consumption gave significant correlations (Table 1) with 64–87% of the variation being accounted for by the animals' weight. A significant increase in oxygen consumption occurs with an increase in temperature giving a Q_{10} of 1.4 (Fig. 5). The slopes of the regression equations at 20°C are not significantly different from those of *Carcinus mediterraneus* but the elevations of the lines are significantly lower for *Pachygrapsus marmoratus*. The slopes of the regressions are similar to those recorded by Roberts (1957) for *Pachygrapsus crassipes*.

Pachygraspsus walking in sea water in the circular respirometer increased its oxygen consumption with increasing walking speed (Fig. 6). Significant correlations were obtained with arithmetic and semilogarithmic models; on the basis of the coefficient of determination the arthimetic model is better. The

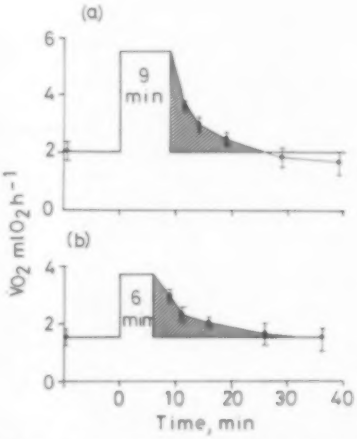

Fig. 4. Changes in oxygen consumption of (a) *Carcinus mediterraneus* and (b) *Pachygrapsus marmoratus* in sea water after a period of exercise. The resting rate of oxygen consumption was measured, and then the animals experienced exercise for the periods shown. Estimates of oxygen consumption during the exercise were made from Figs 3 and 6. Oxygen consumption after the exercise period was compared with the pre-exercise level to give the oxygen debt shown as the shaded area. Experiments were at 20°C, each mean is calculated from four animals.

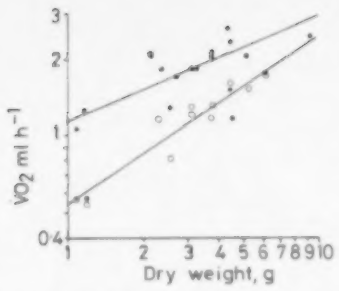

Fig. 5. Relationship between oxygen consumption in sea water to body weight for *Pachygrapsus marmoratus* at rest at 20°C (○) and 30°C (●). The lines are from the regression analysis shown in Table 1.

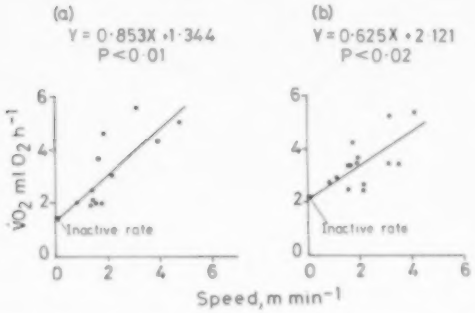

Fig. 6. Rates of oxygen consumption in sea water as a function of walking speed for *Pachygrapsus marmoratus*. Each point is from a single animal and has been calculated for a 5 g dry wt animal. The lines are from the regression analyses shown where Y is oxygen consumption (ml O_2/animal/hr) and X is speed (m/min). The rates of oxygen consumption for inactive animals are not included in the regressions and are taken from Table 1. (a) 20°C, (b) 30°C.

intercepts of the regressions are very similar to the rates of oxygen consumption for inactive animals and are not significantly different from them (Fig. 6).

Comparisons of the regressions at 20 and 30°C indicate that the slope does not change significantly but that the line is significantly higher at 30 than at 20°C.

In order to determine the oxygen debt at 20°C animals were stimulated to walk for a mean time of

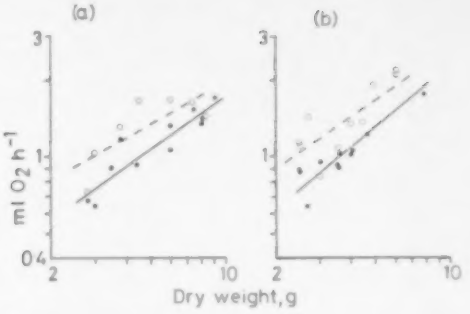

Fig. 7. Relationships between oxygen consumption in air to body weight for *Carcinus mediterraneus* at rest (●) and during activity (○) induced in the respirometer shown in Fig. 1 (b). The lines are from the regression analyses shown in Table 2. (a) 20°C, (b) 30°C.

6.05 ± 1.4 min at a mean speed of 2.89 ± 0.25 m/min, i.e. at about their maximum speed and duration (Table 3). Oxygen consumption for a 5 g dry wt animal walking at this speed was calculated from the speed vs oxygen consumption regression shown in Fig. 6. After the period of walking oxygen consumption declined to pre-exercise levels after 30 min. The amount of the oxygen debt (net debt) was calculated from the shaded area shown in Fig. 4. It amounts to 0.219 ml O_2. The net increase in oxygen consumption during walking is 0.217 ml O_2; the oxygen debt is about 100% of the extra oxygen consumed during walking.

Oxygen consumption in air

The oxygen consumption of *Carcinus mediterraneus* after a 20 min period in air was related to the animal's weight (Fig. 7) (Table 2). Animals were stimulated to maximum activity for 2–3 min in the respirometers after which they were exhausted. The oxygen consumption of these animals is significantly higher than that of inactive animals ($P < 0.01$ at both 20 and 30°C) although there are no significant changes in slope. Activity brings about a 1.3-fold increase in oxygen consumption compared with the inactive rate at both 20 and 30°C. The inactive and active rates of oxygen consumption have Q_{10} values of between 1.2 and 1.3 in air.

Table 2. Regression analyses of dry weight (X, g) and O_2 consumption (Y, ml O_2/hr) for *Carcinus mediterraneus* and *Pachygrapsus marmoratus* in air

Species	Condition	Regression equation	r
Carcinus mediterraneus	20°C Inactive	log Y = 0.696 log X − 0.455	0.913
	Active	log Y = 0.565 log X − 0.265	0.775
	30°C Inactive	log Y = 0.813 log X − 0.449	0.926
	Active	log Y = 0.688 log X − 0.251	0.738
Pachygrapsus marmoratus	20°C Inactive	log Y = 0.751 log X − 0.422	0.880
	Active	log Y = 0.948 log X − 0.185	0.979
	30°C Inactive	log Y = 0.474 log X − 0.128	0.866
	Active	log Y = 0.354 log X + 0.301	0.731

Inactive and active animals were given 20 min in the respirometers before the measurements were begun; active animals were stimulated to activity in the respirometers. Each regression analysis was calculated from at least 15 determinations. r, correlation coefficient.

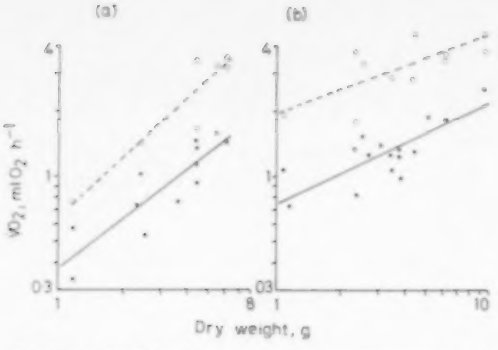

Fig. 8. Relationships between oxygen consumption in air to body weight for *Pachygrapsus marmoratus* at rest (●) and during activity (○). The lines are from the regression analyses shown in Table 2. (a) 20°C, (b) 30°C.

In *Pachygrapsus marmoratus* activity in air brought about a considerable increase in oxygen consumption and activity was maintained in the respirometer for several min (Fig. 8). There are no significant changes in slope of the oxygen consumption vs weight regression during activity and the aerobic expansibilities are 2.4-fold at 20°C and around 2.2-fold at 30°C. The Q_{10} values are around 1.2 in air.

Walking in sea water and air

When the crabs were stimulated to walk in a large tank in sea water they quickly began walking at a constant speed which they maintained until just before they became exhausted (Figs 9 and 10). Just before exhaustion the animals trailed their chelae on the ground. The animals seemed to slow down just before they changed direction. There was very little variation between individuals within a species in the duration and speed of walking in any particular condition (Table 3). *Carcinus mediterraneus* shows a significant increase in walking speed and duration with an increase in temperature in sea water ($P < 0.05$). *Carcinus* has little ability to walk in air; both speed and duration are significantly reduced compared with sea water ($P < 0.05$) and an increase in temperature in air does not improve performance.

Pachygrapsus marmoratus did not walk for more than 6 min in either air or sea water and did not walk as quickly as *Carcinus* (Table 3). What is remarkable about *Pachygrapsus* is its ability to walk in both sea water and air; the duration of walking in air at 20°C is 54% of that in water and at 30°C is not significantly different from that in sea water.

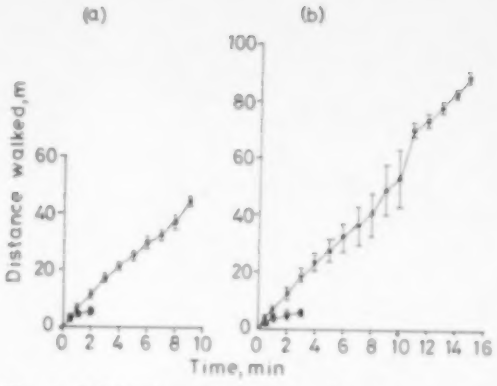

Fig. 9. Relationships between the distance walked and duration of walking for *Carcinus mediterraneus* stimulated to walk at their maximum pace. Each point is a mean (±SE) from 10 animals. Experiments were in sea water (●) or in air (■) at (a) 20°C, (b) 30°C.

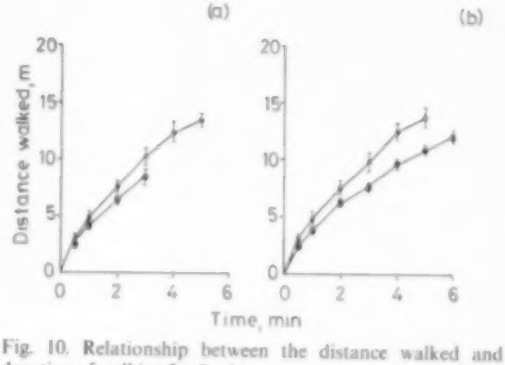

Fig. 10. Relationship between the distance walked and duration of walking for *Pachygrapsus marmoratus* stimulated to walk at their maximum pace. Each point is a mean (±SE) from 10 animals. Experiments were in sea water (●) or in air (■) at (a) 20°C, (b) 30°C.

Aerobic expansibility in sea water and air

The maximum sustainable level of oxygen consumption, which has been used as a measure of maximum aerobic metabolism (Bennett and Licht, 1972) was not measured in the present experiments. The speeds in sea water which generated the highest levels of oxygen consumption in the respirometer could only be sustained for around 5–12 min. Using the mean speeds in sea water from making animals walk at their maximum speeds in an open tank (Table 3) with the oxygen

Table 3. Mean (±SE) duration and speed of walking of *Carcinus mediterraneus* and *Pachygrapsus marmoratus* in sea water and air

Species	Temperature (°C)	Sea water Duration (min)	Sea water Speed (m/min)	Air Duration (min)	Air Speed (m/min)
Carcinus mediterraneus	20	8.58±0.54	4.24±0.23	2.13±0.26	2.85±0.29
	30	12.48±1.65	5.85±0.02	3.15±0.26	1.72±0.10
Pachygrapsus marmoratus	20	4.91±0.92	2.53±0.21	2.66±0.34	2.66±0.30
	30	5.08±0.76	2.61±0.17	5.82±0.54	1.86±0.05

The animals were made to walk at their maximum speed until they could no longer right themselves. Each mean is calculated from between 6 and 14 animals.

consumption vs speed graphs (Figs 3 and 6) allows an estimate of the mean maximum oxygen consumption. It should be noted that some individuals in the respirometer reached speeds slightly above the mean maximum speed recorded in the open tank (comparisons of Figs 3 and 6 and Table 3). Maximum oxygen consumption from walking speed measurements has higher values than those from animals stimulated to activity in the small respirometer and will therefore be used in the estimate of aerobic expansibility. Aerobic expansibility in air can only be estimated from the level of oxygen consumption of active animals in small respirometers (Figs 7 and 8).

In *Carcinus mediterraneus* aerobic expansibility was around 2.1–2.2 in sea water but only 1.3 in air. The aerobic scope can be as high as 3.74 ml O_2/5 g dry animal/hr in water and falls to 0.274 ml O_2/animal/hr in air (Fig. 11). A 5 g dry wt *Pachydrapsus marmoratus* has an aerobic expansibility as much as 2.3 in water and 2.2 in air. The aerobic scope was 1.9 ml O_2/animal/hr in both sea water and air.

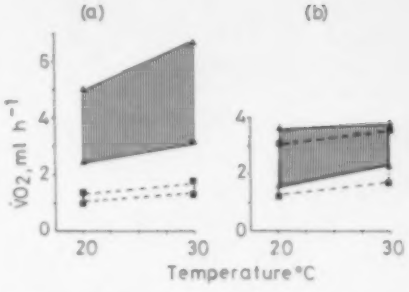

Fig. 11. Rates of oxygen consumption in sea water (▲, shaded) and air (■) as a function of temperature for a 5 g dry wt *Carcinus mediterraneus* (a) and *Pachygrapsus marmoratus* (b). The lower set of symbols for each medium represents resting rates and the upper set of symbols represents the maximum calculated rates of oxygen consumption (Figs 3 and 6 for sea water and Figs 7 and 8 for air).

Circulatory changes during activity in sea water

Post-branchial blood taken quickly by heart puncture from inactive *Carcinus mediterraneus* in sea water at 20°C had high pO_2 values (Table 4) which are above those reported by Taylor and Butler (1978) for *Carcinus maenas* at 15°C in sea water. The haemocyanin was reported to be saturated at a pO_2 of 20 mm Hg in this species, and if the haemocyanin of *Carcinus mediterraneus* is similar, most of the oxygen delivered to the tissues must be supplied from physical solution. The pH values from pre- and post-branchial blood are not significantly different; a similar finding have been found for other crabs (Magnum and Weiland, 1977; Houlihan *et al.*, 1980).

After 4 min of rapid walking in sea water the pO_2 and C_{O_2} values had fallen to low levels so that the venous reserve of oxygen bound to haemocyanin was almost exhausted. Arterial blood saturation also fell. The blood becomes more acid during activity although the mean results are not significantly different from the inactive animal values. The arterial to venous difference fell slightly during activity (Table 4).

Records of heartbeat showed a 14% increase in heart rate between inactive animals and active animals. Heart rate seemed to increase as exercise continued; the measurements given in Table 4 were made after 4 min of activity.

Cardiac output was calculated from the oxygen consumption of an inactive standard sized animal using Table 1 and the arterial to venous difference in oxygen content in Table 4. The result is a cardiac output of 6.03 ml/5 g dry wt animal/min. With the mean resting heart rate this gives a stroke volume of 38.2 μl. When the animals become active the cardiac output rises to 14.6 ml/animal/min and the stroke volume to 81 μl.

Pre- and post-branchial blood samples from *Pachygrapsus marmoratus* had lower pO_2 values than those from *Carcinus mediterraneus* (Table 4). During activity these values fell as with the latter species. During activity heart rate increased by 29% and arterial to venous difference in oxygen content fell slightly. Cardiac output during inactivity was calculated as 6.00 ml/5 g dry wt animal/min giving a stroke volume of 29.5 μl. During activity cardiac output rose to 17.7 ml/animal/min and stroke volume was 67.0 μl. Estimates of heart volume in inactive animals by weighing microfil casts and by blood volume measurements gave similar values to those obtained by calculation.

Some measurements were made on the heart beat of five *Carcinus mediterraneus* which were exposed to air. The mean results showed a 27% decline in heart rate. By contrast *Pachygrapsus marmoratus* showed no change in heart rate after 30 min in air.

Table 4. Mean (+SE) measurements of pO_2, pH and oxygen content (C_{O_2}) from pre- and post-branchial blood from *Carcinus mediterraneus* and *Pachygrapsus marmoratus*

			pO_2 (mm Hg)	pH	C_{O_2} (vol%)	Heart rate (beats/min)
Carcinus mediterraneus	inactive	post	115.0 ± 7.31	7.63 ± 0.02	1.16 ± 0.12	158 ± 15
		pre	14.3 ± 1.52	7.57 ± 0.04	0.49 ± 0.20	
	active	post	45.1 ± 14.2	7.56 ± 0.02	0.73 ± 0.06	180 ± 10
		pre	11.9 ± 3.4	7.49 ± 0.15	0.16 ± 0.02	
Pachygrapsus marmoratus	inactive	post	58.5 ± 10.7	7.69 ± 0.01	0.93 ± 0.07	204 ± 7
		pre	33.0 ± 1.3	7.67 ± 0.02	0.50 ± 0.06	
	active	post	31.0 ± 5.7	7.71 ± 0.04	0.49 ± 0.19	264 ± 39
		pre	11.0 ± 0.06	7.64 ± 0.05	0.16 ± 0.20	

Experiments were carried out in aerated sea water at 20°C. Inactive animals had been undisturbed for 2 hr while active animals had been walking at their maximum speed for 4 min. Results from measurements of heart rate are from inactive animals at the end of the period of activity. Measurements are from at least 5 animals.

DISCUSSION

The results from the measurement of oxygen consumption of the two intertidal decapods used in the present study agree with the patterns of zonation and the characteristics of oxygen consumption in air and water of intertidal gastropods outlined in the introduction. The amphibious *Pachygrapsus marmoratus* respire almost equally well in air and sea water. Using as an index the ratio of aquatic to aerial oxygen consumption of inactive animals, *Pachygrapsus* has ratios of 1:0.82 and 1:0.72 at 20 and 30°C respectively. By contrast, the primarily aquatic *Carcinus mediterraneus* has aquatic to aerial ratios of oxygen consumption of around 1:0.4 at the two temperatures. It is notable that intertidal gastropods show similar aquatic to aerial ratios, with low shore species having ratios of around 1:0.5 and mid shore species having ratios of around 1:1 (Houlihan and Innes, 1982a).

The ratios of aquatic to aerial oxygen consumption obtained in the present study are somewhat different from those found from similar species from previous studies. Burke (1979) records an aquatic to aerial ratio of 1:0.65 for the intertidal *Pachygrapsus crassipes* and the ratios for *Carcinus maenas* range from 1:0.75 (Newell *et al.*, 1972; Wallace, 1972) to 1:1 (Taylor and Butler, 1978). The reasons for these differences are not clear.

When the aquatic to aerial ratios of oxygen consumption are considered from a wide range of intertidal crustacea values range from 1:0.14 for the subtidal *Homarus vulgaris* (Thomas, 1954) to 1:2 for the tropical land crab *Cecarcinus* (Taylor and Davies, 1982) thereby giving some extra support to the hypothesis that the aquatic to aerial ratio may be closely linked to aerial exposure of intertidal crustacea.

Correlations between zonation and aquatic to aerial ratios of oxygen consumption can be drawn not only for the inactive rates but also for the active rates of oxygen consumption. However, when considering aquatic oxygen consumption during activity it is clear that much higher rates of oxygen consumption were obtained from animals stimulated to walk in the larger respirometer than from animals stimulated to apparent maximum and continuous activity in a small respirometer. The active rates of oxygen consumption in air, which were only carried out in the small respirometers, were therefore probably underestimates. However, combining the maximal rates of oxygen consumption from animals walking in sea water and the values for animals active in air reveals that *Carcinus mediterraneus* has a large aerobic expansibility in sea water but a small expansibility in air whereas *Pachygrapsus marmoratus* can increase its oxygen consumption equally in both air and sea water. A very similar pattern of inactive and active rates of oxygen consumption related to zonation has been found for a range of intertidal gastropods (Houlihan and Innes, 1982b).

Another feature of the oxygen consumption of the two decapod species is the relatively temperature independence of the inactive and active rates of oxygen consumption in both species. Responses to temperature were not clearly related to zonation or activity in air or water.

Walking speeds in decapod crustaceans have been divided into short term burst speeds, prolonged speeds leading to fatigue, and sustained speeds that can be maintained almost indefinitely (Wood and Randall, 1981a; Houlihan *et al.*, in preparation). The very fast speeds recorded for *Pachygrapsus transversus* in air (55.2 m/min; Hafeman and Hubbard, 1969) and *Pachygrapsus crassipes* (30 m/min; Burke, 1979) were probably burst speeds leading quickly to fatigue and fuelled primarily by anaerobic metabolism. The walking speeds used in the present study can be generally classed as prolonged speeds which in crabs are associated with an increase in oxygen consumption, an accumulation of an oxygen debt, and a duration of around 10 min (Houlihan *et al.*, in preparation). However, walking of *Carcinus mediterraneus* in air was associated with only a slight increase in oxygen consumption and a short duration.

An increase in temperature brought about a significant increase in duration of walking and walking speed of *Carcinus mediterraneus* in sea water and the duration of walking of *Pachygrapsus marmoratus* in air. Burke (1979) reports an increase in burst speeds with some individuals of *Pachygrapsus crassipes* with an increase in temperature and this has also been found for a number of invertebrates (Houlihan and Innes, 1982b).

Both *Carcinus mediterraneus* and *Pachygrapsus marmoratus* showed an increase in oxygen consumption after walking in sea water for 5 min. Experiments lasting 10 min gave rates of oxygen consumption which were not significantly different from the shorter duration experiments. These two species together with *Callinectes sapidus* (Booth *et al.*, 1982) show a rapid increase in oxygen consumption with activity and are in contrast to the reports from running in terrestrial crabs where steady states of oxygen consumption were not reached after 10 to 20 min (Herreid *et al.*, 1979; Full and Herreid, 1980; Wood and Randall, 1981a). These differences require further investigation.

With increasing walking speed the oxygen consumption in sea water of both *Carcinus mediterraneus* and *Pachygrapsus marmoratus* increases linearly. A linear relationship between speed and oxygen and consumption has been found from a number of studies on pedestrian locomotion (Herreid, 1981) and crawling in gastropods (Houlihan and Innes, 1982b). Unlike many of these studies the results from the present study reveal no significant differences between the measured inactive rates of oxygen consumption and the Y intercepts of the linear model of walking speed and oxygen consumption.

The maximum rates of oxygen consumption reached by *Carcinus mediterraneus* and *Pachygrapsus marmoratus* during walking in sea water were around 3–4 times above the inactive rates and these results agree with the suggestion that the highest levels of oxygen consumption that can be maintained by walking arthropods for any length of time are about 3–7 times higher than the inactive rates (Herreid, 1981), even in swimming *Callinectes sapidus* oxygen consumption increased by only 2.6 times (Booth *et al.*, 1982).

Fast prolonged speeds in sea water resulted in oxygen debts of between 40 and 100% of the net increase in oxygen consumption during exercise and these debts were repayed within 20 min. These results are in marked contrast to debts of 1100% of the net

increase taking up to 7 hr to repay reported from running in land crabs (Wood and Randall, 1981a).

The accumulation of an oxygen debt during exercise raises the question of whether the oxygen consumption during exercise can be related to performance. As an index of performance, duration of walking seems more useful than speed; as speed did not vary significantly between different conditions. A plot of aerobic scope, i.e. the difference between the maximum levels of oxygen consumption and the inactive rate (Bennett and Licht, 1972) and duration reveals that a correlation between performance and oxygen consumption does exist (Fig. 12). *Carcinus mediterraneus* in water walks the farthest and has the largest aerobic scope. The smallest scope, for *Carcinus mediterraneus* in air correlate with the shortest duration of walking. *Pachygrapsus marmoratus* lies between these two extremes.

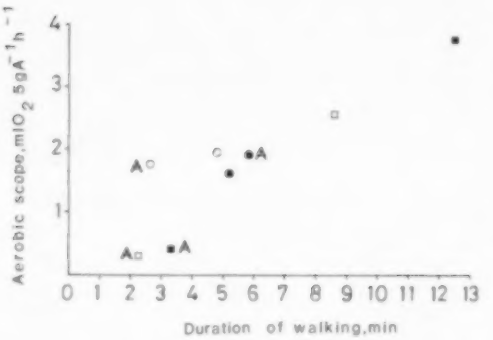

Fig. 12. Relationship between duration of walking at fast prolonged speeds and the aerobic scope at these speeds. The points are from *Carcinus mediteranneus* (□) and *Pachygrapsus marmoratus* (○) in sea water and air (A) 20°C (open symbols) and 30°C (closed symbols). Aerobic scope was calculated for a 5 g dry wt animal as the difference between the resting rate and the oxygen consumption at the mean walking speeds shown in Table 3.

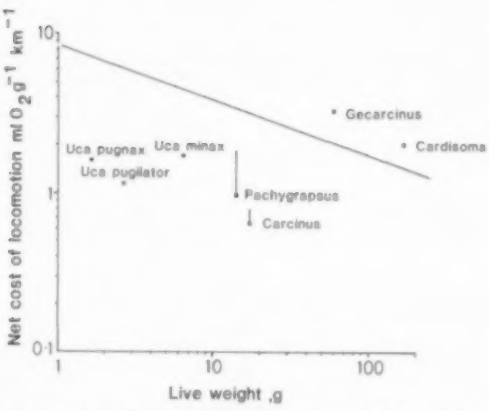

Fig. 13. Net cost of transport vs body wt. The solid line is from the relationship for running vertebrates and ants (Jensen and Holm-Jensen, 1980). The values for crabs in air are cumulative net costs including oxygen debts (Herreid, 1981). The values for *Carcinus mediterraneus* and *Pachygrapsus marmoratus* are from 20°C and the values including the oxygen debts are shown as vertical lines.

The total cost of transport, i.e. the metabolic cost of transporting a unit of mass over a unit of distance (Taylor *et al.*, 1970), declines rapidly with increasing speed and approaches a minimum cost at speeds around 4 m/min. Total aerobic cost of transport in sea water of *Carcinus mediterraneus* walking at 5 m/min was 1.2 ml O_2/g live wt/km and 1.4 ml O_2/g/km at 20 and 30°C respectively. Comparable figures for *Pachygrapsus marmoratus* at 4 m/min were around 1.4 ml O_2/g/km at both 20 and 30°C. Net costs of transport are frequently used for comparisons of costs between different species and are calculated as the difference between the total costs and the resting rate of oxygen consumption. The net costs are shown in Fig. 13 in relation to live weight. The net costs do not change significantly between 20 and 30°C for *Carcinus* but the fall from 0.95 to 0.72 ml O_2/g/km in *Pachygrapsus* between 20 and 30°C.

If the net oxygen debts shown in Fig. 4 are added to the net aerobic costs, the net "cumulative costs" are obtained (Herreid, 1981). Included in Fig. 13 are net cumulative costs of transport for some decapods in air from Herreid *et al.* (1979) and Herreid (1981). Also included is the line relating aerobic cost of transport and body weight for walking ants and mammals (Jensen and Holm-Jensen, 1979). The decapod values appear to be rather similar. The two *Carcinus* species and *Pachygrapsus* are walking in sea water whereas the other species are walking in air. The decapods weigh over 10 times less in water than in air but this does not seem to greatly reduce the net costs of transport when compared with terrestrial species.

The basic strategy of both *Carcinus mediterraneus* and *Pachygrapsus marmoratus* for increasing oxygen uptake during exercise in sea water appears to be the same as that adopted by the land crab *Cardisoma* and the marine crabs *Cancer* and *Callinectes* (Wood and Randall, 1981b; McMahon *et al.*, 1979; Mangum and Weiland, 1975 respectively). There is a fall in arterial and venous pO_2, and although dissociation curves were not determined in the present study, this presumably brings oxygen transport onto the steep part of the haemocyanin dissociation curve. The arterial to venous difference remains the same so that with the small increase in heart rate an increase in stroke volume by 100% is necessary to account for the increase in oxygen consumption. The heart rate increases during exercise of around 15–30% are similar to those found for *Cardisoma* (Wood and Randall, 1981a) and *Cancer* (McMahon *et al.*, 1979).

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AN ELECTROGENIC COMPONENT IN THE MEMBRANE POTENTIAL OF *TETRAHYMENA*

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Abstract—1. The average resting potential of *Tetrahymena vorax* was -24.15 ± 0.78 mV ($n = 139$).

2. The addition of Ouabain at 10^{-5} M resulted in a decrease in the membrane potential of approximately 30%.

3. The response of the resting potential to changes in temperature was measured to determine whether it was dependent upon an active component. The response of the membrane potential to cooling was slower than the response to warming, although there was no significant difference in overall magnitude between the two types of response.

4. The Q_{10} value for the membrane potential changes in control solution (Na^+-free) was 1.22 ± 0.03 ($n = 25$). This is significantly greater ($P < 0.01$) than the value predicted by electrodiffusion theory (1.035) and suggests that there is some Na^+ independent active transport involved in the maintenance of the membrane potential.

5. In control solution containing 20 mM Na^+ the Q_{10} value was 1.44 ± 0.6 ($n = 16$). This is significantly greater ($P < 0.001$) than the response in control solution alone, indicating that the Na^+ ion may be involved in an electrogenic transport mechanism.

6. Ouabain significantly ($P < 0.01$) reduced the Q_{10} value in Na^+ solution from 1.44 ± 0.06 ($n = 16$) to 1.09 ± 0.01 ($n = 6$). The reduction of the Q_{10} value in control solution (Na^+-free) from 1.22 ± 0.03 ($n = 25$) to 1.14 ± 0.01 ($n = 6$) was not significant at the 1% level ($0.25 > P > 0.1$). This suggests that an electrogenic Na^+–K^+ ATPase contributes to the membrane potential in Tetrahymena. The hyperpolarising effect of this ion pump may stabilise behaviour by reducing the number of spontaneous regenerative depolarisations.

7. Screening of localised electrical events by high resistance pathways in Tetrahymena and Paramecium is discussed. These pathways may be mediated by specialised membranes with reduced permeability so that there is considerable electrical heterogeneity among the membranes of the cell. Thus it is suggested that membrane constants obtained for Tetrahymena, and possibly mammalian neurones also, should not be considered as the product of simple isopotential systems, but rather as the complex average of many electrically distinct components.

INTRODUCTION

Active transport of solutes may be classified as a primary or secondary process. Primary active transport involves the coupling of the passage of solutes across the membrane to the advancement of a chemical reaction. Examples of this type of "pump" are the Na^+–K^+, Mg^{2+} activated ATPase (Skou, 1957) and the Ca^{2+}-ATPase of the sarcoplasmic reticulum (Hasselbach and Mackinose, 1963). Secondary active transport involves the exchange of two solutes. One of these flows down its concentration gradient and drives the other against its gradient. The Na^+–Ca^{2+} exchange in cardiac muscle (Reuter and Seitz, 1968) typifies this class of pump.

The number and charge of ions exchanged across the membrane may not be equal. If the net charge transferred across the membrane is zero, the pump is said to be electroneutral, if not, the pump is called electrogenic. An electroneutral pump can indirectly create a resting potential because of the selective permeability of the membrane to different ions (Goldman, 1943; Hodgkin and Katz, 1949) and the presence within the cell of non-diffusible anions (Donnan, 1911). However an electrogenic pump can make a further contribution to the membrane potential by directly separating charges across the membrane. If the flow of charge is small then the immediate contribution of the pump will be small and its effect will be noticed as a long term change in ion concentrations across the membrane. Such is the case in the squid axon (Hodgkin, 1951), and the behaviour of the membrane potential in this cell is successfully described by electrodiffusion theory (Goldman, 1943; Hodgkin and Katz, 1949; Hodgkin and Huxley, 1952a, b; Hodgkin *et al.*, 1952). However, if the current flow across the resistance of the membrane is at any given instant larger, it may become significant enough to make a direct and immediate contribution to the membrane potential. Electrogenic pumps of this type have been described in several preparations, including *Helix aspersa* (Kerkut and Thomas, 1965), *Anisodoris* (Marmor and Gorman, 1970), *Acetabularia* (Saddler, 1970), heart (Délèze, 1960) and mitochondria (Mitchell, 1961).

A membrane potential which includes an immediate electrogenic component will respond differently to changes in temperature (Ling and Gerard, 1949; Kerkut and Ridge, 1961) when compared with one which is essentially the result of simple diffusion (Goldman, 1943; Hodgkin, 1951). An electrogenic component in a membrane potential would result in a greater potential response to changes in temperature than would be expected from calculations based on electrodiffusion theory. The proportional change in

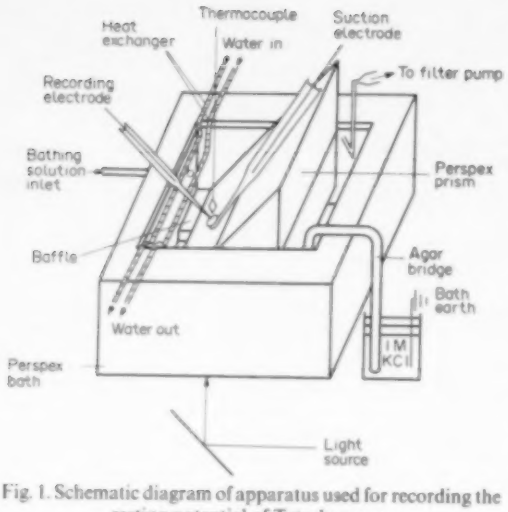

Fig. 1. Schematic diagram of apparatus used for recording the resting potential of Tetrahymena.

membrane potential for a 10°C change in temperature can thus be used as an indication of the dependence of a resting potential upon metabolism and electrogenic components (Lund and Moorman, 1931; Kerkut and York, 1969).

The present work defines the normal range of resting potentials for *Tetrahymena vorax*. The Q_{10} value for membrane potential in control solution and in the presence of Ouabain and/or Na^+ is determined in order to investigate whether there is an immediate, active component in the resting potential of Tetrahymena.

MATERIALS AND METHODS

Intracellular recordings were obtained from *Tetrahymena vorax* which had been grown axenically and harvested at 20°C as described previously (Connolly and Kerkut, 1981). The apparatus used for recording from the cells is shown in Fig. 1. The Perspex bath normally contained control solution (1 mM KCl, 1 mM $CaCl_2$, 0.1 mM EDTA and 1 mM Tris–HCl pH 6.8). A 2 cm^3 syringe was used to introduce different solutions into the bath through a narrow inlet. Gentle, steady pressure would ensure that the flow of solution directed towards the preparation by the baffle did not release the cell and did not exceed the capacity of the constant head device behind the prism to remove the solution. When methylene blue dye was added to the bath it was found that the bath volume of 1.25 cm^3 was completely cleared with 5 sec. The preparation was illuminated from the base of the bath and viewed through a stereoscopic microscope mounted in the same plane as the observer in Fig. 1. This elevation gives dark field illumination and facilitates 3-dimensional perception.

The temperature of the bathing solution was altered by passing hot or cold solutions through two glass tubes located in the bath which acted as heat exchangers. Temperature measurements were made using a thermocouple placed in the bathing solution and adjacent to the cell. The temperature reading was displayed on a temperature meter and recorded on a Watanabe pen recorder.

The temperature sensitivity of the electrodes was measured before and after recording from the cell. Any electrodes which showed a potential drift of greater than ±0.5 mV per 10°C temperature change were rejected. The temperature measuring system was accurate to within ±0.2°C within the temperature range investigated (13–27°C). Voltage measurements were made using a digital voltmeter and recorded on a Watanabe pen recorder, but because of the level of background potential fluctuations in Tetrahymena, are probably only accurate to ±0.25 mV.

Treatment of results

Calculation of Q_{10}. The Q_{10} value describes the proportional change in the magnitude of a parameter for a 10°C change in temperature. If membrane potential is a function of the absolute temperature (T) only, then the ratio of the values of potential at two different temperatures will equal the ratio of the absolute value of those temperatures.

Considering a 10°C change about 19°C (292 K, approximately the average temperature at which the experiments were performed) then the temperature ratio (Q_{10} value) = 297/287 = 1.035. This Q_{10} value would then be expected to represent the ratio of the membrane potentials at 297 and 287 K for a non-electrogenic resting potential.

Most experiments did not span exactly the 10°C between 14 and 24°C. For all cells, a value for the change in mV/°C was first calculated. This result was then extrapolated about the average potential to give a Q_{10} value. An example is given below:

T_1: 17°C, T_2: 23°C, $\bar{T}$: 20°C, mV_1: 23.5, mV_2: 26.5, $\overline{mV}$: −25, $\Delta mV/\Delta T$ = 0.5 mV°C^{-1}. Thus there is a change of 0.5 mV for a 1°C change in temperature. This is then extrapolated to a 5 mV change in resting potential for a 10°C change in temperature about −25 mV, which gives a Q_{10} value of 27.5/22.5 = 1.22. Where appropriate, the Student's t-tests for paired and independent samples were used to determine the significance of the results.

RESULTS

Control resting potential

The average resting potential of 139 cells in control solution was −24.15 ± 0.78 mV. The range was from −10 to −44 mV if cells undergoing regenerative hyperpolarisations were excluded from the sample. A histogram depicting the frequency distribution of resting potential is provided in Fig. 2. The average input resistance of 26 cells in control solution was 47.8 ± 2.9 MΩ.

The effect of Ouabain on the membrane potential

The effects of Ouabain (Strophanthin-G) addition to cells in control solution is shown in Table 1. In all but

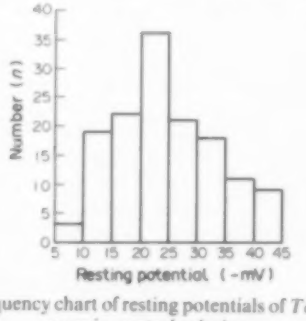

Fig. 2. A frequency chart of resting potentials of *Tetrahymena vorax* in control solution.

Abbreviations
$[X^+]_i$ refers to the intracellular concentration of species X^+.
$[X^+]_o$ refers to the extracellular concentration.
C-V is the contractile vacuole.
"Na^+ solution" refers to control solution containing an added 20 mM NaCl.

Table 1. The effect of Ouabain addition on resting potential

Ouabain M	Response time (sec)	$R.P._1$ (mV)	$R.P._2$ (mV)	Δ mV	% change	n
10^{-7}	90	−18	−15	+3	16.7	1
10^{-6}	50 ± 10	−25	−16 ± 1	+9 ± 1	36.0 ± 4	2
10^{-5}	94 ± 9.3	−27.5 ± 3.4	−18.2 ± 3.2	+9.3 ± 1.1	34.8 ± 4.6	5
5×10^{-5}	66.7 ± 14.5	−24.7 ± 1.7	−18.3 ± 4.4	+6.4 ± 1.9	27.1 ± 8.9	3
10^{-4}	125	−25	−10	+15	60.0	1

one cell there was a gradual depolarisation which took an average time of 82 ± 8 sec ($n = 12$) to attain the maximum response. An example of such a response is shown in Fig. 3A. In one instance at 10^{-6} M there was no apparent response to Ouabain. The maximum response at 10^{-7} M may not be complete whereas the maximum response at 10^{-4} M probably involves a non-specific component.

Attempts to reverse completely the effects of Ouabain were unsuccessful unless washing followed almost immediately. In the results quoted in Table 1 washing was carried out 30 sec after addition of the Ouabain. If this was not done the cells would continue to depolarise at a reduced rate and eventually become unviable. The average input resistance of the cells in 10^{-5} M Ouabain as 45 ± 14.1 MΩ ($n = 5$). This is not significantly different from the input resistance in control solution alone ($P > 0.6$) which is 47.8 ± 2.9 MΩ ($n = 26$). Thus it would seem that Ouabain added in this way is not causing a non-specific reduction in membrane resistance. The average % change in resting potential after addition of 10^{-5} M and 5×10^{-5} M Ouabain was 31.9 ± 4.3% ($n = 8$). This may be an approximate indication of the contribution of electrogenic pumping by a Na^+–K^+ ATPase to the resting potential of these cells.

Resting potential changes in control solution

Figure 4 shows examples of the response of the resting potential of Tetrahymena to temperature change. This response typically followed temperature changes with a time lag of 5–10 sec. Figures 4(a) and (b) contrast the range of the responses. Figures 4(a) and (c) demonstrate that the responses were reversible, with no significant differences between paired cooling and warming responses in individual cells or between the independent cooling and warming responses given in Table 2. It is interesting to note, as Kerkut and Ridge (1961) observed in crab, insect and frog muscles, that the resting potential is slower to react to cooling than to warming.

This raised the question as to whether it was reasonable to extrapolate individual results to give a Q_{10} for the whole temperature range. This assumes that the rate of change of resting potential for a 1°C change in temperature is constant over the range of temperature studied. Responses in Table 2 whose mean temperature during the experiment was greater than the mean of the whole group (18.7°C) were compared with those responses whose mean temperature was lower than the group mean. There was found to be no significant difference between these groups. The assumption therefore seems justified for these cells over the temperature range studied.

The average change in resting potential for a 10°C change in temperature about 18.7°C was 4.2 ± 0.06 mV ($n = 25$). This is significantly greater than the value 0.76 mV which would be predicted by the Nernst equation ($P < 0.01$). The experimental Q_{10} value of 1.22 ± 0.03 is also significantly larger than the theoretical value ($P < 0.01$). These are strong indications of a metabolic contribution to the membrane potential of *Tetrahymena vorax*.

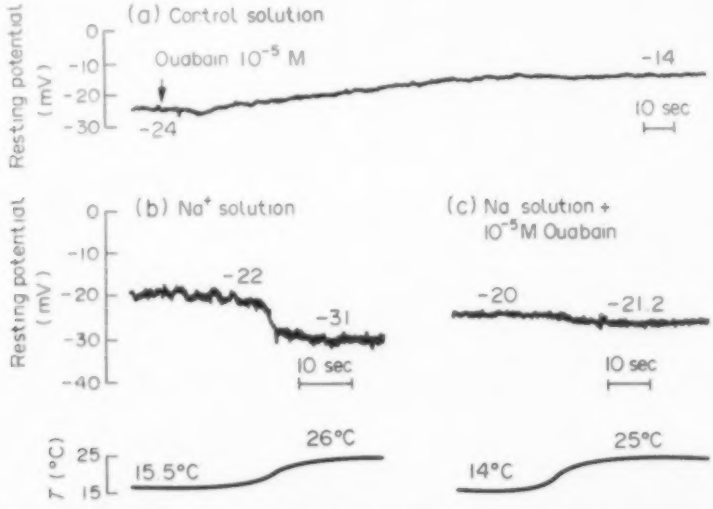

Fig. 3. The effects of Ouabain addition on the resting potential and its response to temperature changes.

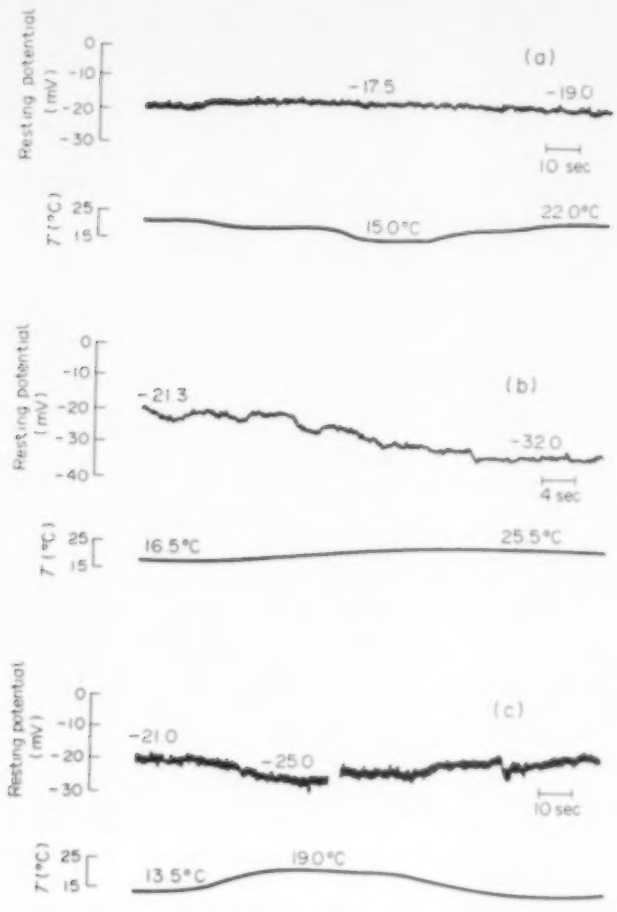

Fig. 4. Examples of resting potential responses to temperature change in control solution.

Table 2. Temperature and resting potential changes in control solution

	R.P.$_1$ (mV)	R.P.$_2$ (mV)	$\overline{R.P.}$ (mV)	T_1 (°C)	T_2 (°C)	$\bar{T}$ (°C)	$\frac{\Delta \text{ mV}}{\Delta \text{ °C}}$	Q_{10}
	−15.0	−14.5	−14.15	19.0	15.0	17.0	0.125	1.09
	−15.0	−13.7	−14.35	20.0	16.0	18.0	0.33	1.26
	−27.0	−22.3	−24.6	19.5	13.5	16.5	0.78	1.38
	−33.0	−32.3	−32.7	20.0	14.0	17.0	0.12	1.04
	−23	−17.3	−20.2	22.0	15.0	18.5	0.96	1.62
	−40	−38	−39	18.0	13.5	15.75	0.44	1.12
	−17.5	−19.0	−18.2	15.0	22.0	18.5	0.22	1.13
	−24	−21.3	−22.7	19.0	13.5	16.25	0.49	1.24
	−18	−16.1	−17.1	25.0	16.0	20.5	0.21	1.13
	−19	−17.5	−18.2	20.0	15.0	17.5	0.3	1.18
	−21	−25	−23	13.5	19.0	16.25	0.73	1.38
	−18	−22.3	−20.2	13.0	24.0	18.5	0.39	1.21
	−20	−18	−19.0	21.0	13.5	17.25	0.27	1.15
	−22	−22.7	−22.35	16.0	20	18.0	0.18	1.08
	−15.3	−18.0	−16.7	16.0	26	21.0	0.27	1.18
	−21.4	−23.6	−22.5	16.2	25	20.6	0.25	1.18
	−19	−21.7	−20.35	13.5	24	18.75	0.26	1.14
	−18	−22.2	−20.1	16.0	23	19.5	0.6	1.35
	−30	−36	−33	16.0	24.5	20.3	0.7	1.28
	−26	−31	−28.5	16.5	25.5	21.0	0.56	1.22
	−21.3	−32	−26.5	16.5	24	20.25	1.43	1.74
	−22	−20	−21.0	23	15	19.0	0.25	1.13
	−22.3	−18.9	−20.6	24	13.5	19.75	0.32	1.17
	−22.7	−23.2	−22.95	20	24	22	0.12	1.05
	−23	−21	−22	24	16	20	0.25	1.12
n			25			25	25	25
$\bar{x}$			−22.4			18.7	0.42	1.22
SE			1.2			0.35	0.06	0.03

Table 3. Temperature and resting potential changes in Na^+ solution

	R.P.$_1$ (mV)	R.P.$_2$ (mV)	$\overline{R.P.}$ (mV)	T_1 (°C)	T_2 (°C)	$\bar{T}$ (°C)	$\frac{\Delta\ mV}{\Delta\ °C}$	Q_{10}
	−19	−14	−16.5	17.5	10	13.75	0.67	1.51
	−17	−18.8	−17.9	16	25	20.5	0.2	1.12
	−18	−17.3	−17.7	17	13	15	0.175	1.11
	−23.5	−19	−21.25	25.5	15.5	20.5	0.4	1.21
	−11	−19	−15	14	23	18.5	0.73	1.65
	−16	−14	−15	16.5	13	14.8	0.57	1.47
	−14	−22.5	−18.25	13	25	19.0	0.71	1.48
	−13	−23.5	−18.25	16.5	25.5	21.0	1.17	1.94
	−16	−11	−13.5	20	14	17	0.83	1.89
	−22	−31	−26.5	15.5	26.0	20.75	0.86	1.39
	−28	−21	−24.5	23	15	19.0	0.88	1.44
	−21	−26.5	−23.75	15	25	20	0.55	1.26
	−15.5	−12.2	−13.8	20	15	17.5	0.67	1.64
	−18	−16.0	−17.0	20	15	17.5	0.34	1.22
	−30	−37.2	−33.6	17	27	22	0.72	1.24
	−12	−17	−14.5	17.5	24	20.75	0.67	1.47
n			16			16	16	16
$\bar{x}$			−19.2			18.6	0.63	1.44
SE			1.4			0.6	0.07	0.06

Temperature and resting potential changes in Na^+ *solution*

Twenty millimoles of NaCl was added to control solution in order to determine whether Na^+ is involved in a metabolic contribution to the resting potential. Chloride ions are assumed to be passively distributed (Dunham and Child, 1961). The results of sixteen independent responses are summarised in Table 3 and an example is shown in Fig. 3(b).

The mean resting potential of cells in this solution is lower than that established for 139 cells in control solution ($P < 0.01$). It is therefore probable that Na^+ contributes an electrodiffusion potential to the total membrane potential. The Q_{10} value of membrane potential responses to temperature changes in Na^+ solution was 1.44 ± 0.06 ($n = 16$), a significantly greater response than in control solution ($P < 0.001$). The Q_{10} value is also greater than that predicted by electrodiffusion theory ($P < 0.001$). The frequency distribution histogram (Fig. 5a) illustrates the shift in the modal classes of potential responses from 0.1 to 0.4 mV/°C in control solution of 0.6–0.9 mV/°C in Na^+ solution. The frequency distribution of Q_{10} values (Fig. 5b) shows a similar shift. This increase in potential responses and Q_{10} values suggests that Na^+ is involved in an electrogenic component of the resting potential of Tetrahymena.

The effect of Ouabain on the membrane potential response to temperature change

The addition of Ouabain at 10^{-5} M resulted in a marked reduction of the membrane potential changes associated with temperature changes. To illustrate this a typical membrane potential change in Na^+ solution (Fig. 4b) is compared with that in Na^+ solution containing 10^{-5} M Ouabain (Fig. 4c). Tables 4 and 5 summarise the results obtained in cells in control and Na^+ solution containing 10^{-5} M Ouabain.

In control solution with Ouabain, the Q_{10} value is 1.14 ± 0.06 ($n = 6$) compared to 1.22 ± 0.03 ($n = 25$) in control solution alone. This reduction is not significant at the 1% level ($0.25 > P > 0.1$). However the reduction by Ouabain of the Q_{10} value in Na^+ solution from 1.44 ± 0.06 ($n = 16$) to 1.09 ± 0.01 ($n = 6$) is

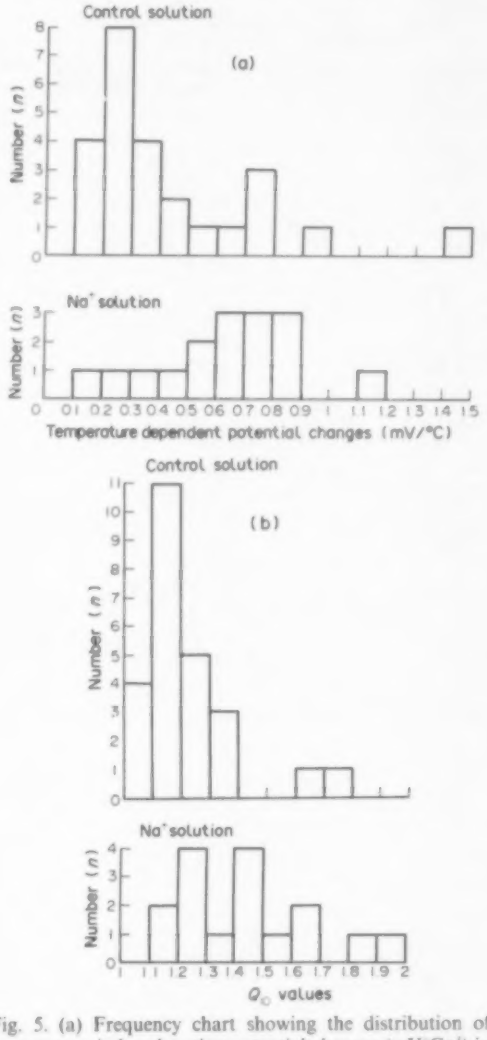

Fig. 5. (a) Frequency chart showing the distribution of temperature induced resting potential changes (mV·°C^{-1}) in control solution and with 20 mM NaCl added. (b) Frequency chart showing distribution of Q_{10} values obtained in control solution and Na^+ solution.

Table 4. Temperature and resting potential changes in control solution containing 10^{-5} M Ouabain

	R.P.$_1$ (mV)	R.P.$_2$ (mV)	R.P. (mV)	T_1 (°C)	T_2 (°C)	$\bar{T}$ (°C)	$\frac{\Delta\ mV}{\Delta\ °C}$	Q_{10}
	−15	−15.3	−15.15	16	19	17.5	0.10	1.07
	−10	−9.0	−9.5	18	12	15.0	0.16	1.18
	−14.5	−13.5	−14.0	17	13.5	15.25	0.29	1.23
	−13.5	−13.0	−13.25	15	12	13.5	0.17	1.14
	−13.0	−14.5	−13.7	16	22	19	0.25	1.20
	−30.9	−31.7	−31.3	20	28	24	0.10	1.03
n			6			6	6	6
$\bar{x}$			−16.2			17.4	0.18	1.14
SE			3.1			1.6	0.03	0.01

significant ($P < 0.01$). The reduction of Q_{10} values by Ouabain suggests that a Na^+–K^+ ATPase may contribute electrogenically to the membrane potential in Tetrahymena. The increased significance of this reduction in the Na^+ solution as compared to control solution further implicates Na^+ as one of the ions involved in electrogenic transport. Despite the action of Ouabain the potential responses in Tables 4 and 5 were still significantly greater than the values predicted by electrodiffusion theory (control solution and Ouabain, $P < 0.001$, Na^+ solution and Ouabain, $P < 0.01$). Thus Tetrahymena may also have electrogenic mechanisms which are not sensitive to Ouabain.

DISCUSSION

In Nature, free living freshwater ciliates such as Tetrahymena occupy a wide variety of habitats which constantly present osmotic and ionic challenges to the internal environment of these cells. In carrying out the processes of feeding, digestion, pinocytosis and osmoregulation Tetrahymena must interact with an uncontrolled external medium, unlike the more protected excitable cells in mammals. Thus it may be expected that Tetrahymena should have powerful and rapidly responsive mechanisms for maintaining the ionic gradients across its membranes.

This would also be expected of Paramecium, a closely related ciliate. The electrical properties of Paramecium (Naitoh and Eckert, 1968, 1972; Kung and Saimi, 1982) are very similar to those of Tetrahymena (Connolly and Kerkut, 1981, 1983). Paramecium has a resting potential of approximately −30 mV in a solution of the same ionic composition as the control solution used in this present work; while Tetrahymena has a resting potential of 24.2 ± 0.8 (n = 139). The input resistance of *Paramecium caudatum* was approximately 64 M (Naitoh and Eckert, 1968) which Machemer and De Peyer (1977) calculate to be equivalent to a specific membrane resistance of $7 \times 10^4 \Omega\ cm^2$. Using the same method of calculation, which is based on the Fortner (1925) formula for surface area, *Tetrahymena vorax*, with an input resistance of 47.8 ± 2.9 MΩ (n = 26), gives a specific membrane resistance of $3.2 \times 10^4\ \Omega cm^2$. These resistances are a factor of 10 greater than more specialised cells such as *Helix pomatia* (Meves, 1968). The similarity between Tetrahymena and Paramecium implies that the conclusions drawn from this present work on Tetrahymena may have a general application to Paramecium.

The effects of Ouabain addition at 10^{-5} M were similar to those observed in other preparations such as snail neurons (Gorman and Marmor, 1970). The concentration used ensured that any reserve capacity (Akera *et al.*, 1981) of a Na^+–K^+-ATPase would be inhibited but that non-specific conductance increases associated with greater Ouabain concentrations (Garrahan and Rega, 1972) would be avoided. The fact that there was no significant reduction in membrane resistance after Ouabain addition supports the assumption that Ouabain is mainly acting in a specific manner. The average % change in resting potential induced by 10^{-5} M Ouabain was 34.8 ± 4.6 (n = 5), and at 5×10^{-5} Ouabain, the change was 27.1 ± 8.9% (n = 3). An average of these results gives 31.9 ± 4.6%. Thus as much as 30% of the resting potential in Tetrahymena may be due an electrogenic Na^+–K^+-ATPase.

Ouabain is a specific inhibitor of the Na^+–K^+ (Mg^{2+})-ATPase (Schatzmann, 1953) and its associated

Table 5. Temperature and resting potential changes in control solution containing 20 mM Na Cl and 10^{-5} Ouabain

	R.P.$_1$ (mV)	R.P.$_2$ (mV)	R.P. (mV)	T_1 (°C)	T_2 (°C)	$\bar{T}$ (°C)	$\frac{\Delta\ mV}{\Delta\ °C}$	Q_{10}
	−20	−21.2	−20.6	14	25	14.5	0.2	1.10
	−26	−25.2	−25.6	24	20	22	0.2	1.08
	−12.5	−14.0	−13.25	15	25	20	0.15	1.12
	−25.5	−24.5	−25.0	22	15	18.5	0.14	1.06
	−18.2	−17.0	−17.6	21	16	18.5	0.22	1.13
	−29	−28	−28.5	22	16	19	0.16	1.06
n			6			6	6	6
$\bar{x}$			−21.8			18.8	0.18	1.09
SE			2.3			1.0	0.01	0.01

phosphatase activity (Inturrisi and Titus, 1970). However Andrus and Giese (1963) found that 10^{-3} M Ouabain did not affect the restoration of Na^+ and K^+ in Tetrahymena which had been exposed to cold, although this concentration of Ouabain might be expected to produce some non-specific effects. Conner *et al.* (1963) did find an Mg^{2+} dependent ATPase in *Tetrahymena pyriformis* whose activity could be enhanced by Na^+ and K^+ by 10–15%. However this enzyme was not sensitive to Ouabain. Baugh *et al.* (1976) found no evidence of the Na^+–K^+(Mg^{2+})-ATPase in ciliary preparations from Tetrahymena, nor Doughty (1978) in Paramecium. Dentler (1977) detected a Na^+–K^+(Mg^{2+})-phosphatase in the ciliary basal bodies of Tetrahymena, but not an ATPase. These negative results are not conclusive however, since the most probable site for a Na^+–K^+(Mg^{2+})-ATPase, the contractile vacuole (CV), has not been investigated.

A Na^+–K^+(Mg^{2+})-ATPase has been identified in *Entamoeba invadens* (McLaughlin and Meerovitch, 1975). Furthermore, Boggs and Wade (1972) in a study on the anterior vacuole of *Paramecium multimicronucleatum*, found that Ouabain slowed the activity and doubled the diameter of the CV. The nature of the mechanism producing these effects is uncertain, but it does suggest that a Na^+–K^+(Mg^{2+})-ATPase may be present in the CV region.

The use of temperature changes to determine whether there is an active component in a membrane potential assumes parameters such as membrane permeability, resistance and internal ion concentrations remain constant over the temperature range investigated. The fact that there is no significant difference between results in Table 2 whose mean experimental temperature was above the average for the whole group and those below it suggests that if there are changes in cell properties these changes must be linear. Furthermore Ouabain specifically reduced membrane potential and Q_{10} values. If the potential change induced by temperature was solely due to a change in a membrane constant Ouabain at the concentration used would not affect it. In Anisoderis (Marmor, 1971) and Aplysia (Russell and Brown, 1972) $pNa^+ : pK^+$ increases with warming. This would be a depolarising influence if the change occurred in Tetrahymena, yet an increase in the hyperpolarising response is observed upon warming in 20 mM Na^+. Cooling Tetrahymena from 25 to 6°C reduces $[K^+]_i$ by 50% but increases $[Na^+]_i$ 2-fold (Andrus and Giese, 1963). Thus the effects of changes in internal ion concentration on membrane potential tend to cancel each other out. Overall, temperature dependent changes in electrophysiological properties would oppose metabolic rate changes in their effect on membrane potential. This is best illustrated in Anisodoris (Gorman and Marmor, 1970) and Aplysia (Carpenter, 1970; Marchiafava, 1970) where the application of Ouabain unmasks changes in membrane parameters and the hyperpolarising effect of warming is reversed to a depolarising effect. In conclusion, it can be said that non-metabolic temperature dependent changes contribute little or more probably detract from the potential changes observed in these experiments. Therefore monitoring the response of the membrane potential to temperature changes is a useful indication of the importance of metabolism in the immediate maintenance of resting potential.

The Q_{10} values obtained for Tetrahymena by the above experimental approach in control ($Q_{10} = 1.22$) and Na^+ solutions ($Q_{10} = 1.44$) were clearly and significantly greater than electrodiffusion theory alone would predict ($Q_{10} = 1.035$). This is also the case in Paramecium (Yamaguchi, 1980) where a 10°C change in temperature gave a 2 mV change in potential ($Q_{10} = 1.07$). However the potential measurements were made some time after the temperature changes so that adaptation may have reduced the potential change observed. These results imply that there is an electrogenic contribution to the membrane potential. The enhancement of the Q_{10} values by Na^+ addition from 1.22 to 1.44 suggests that Na^+ is involved in electrogenic transport. The significant reduction of the Q_{10} value in Na^+ solution ($1.44 \rightarrow 1.09$) by Ouabain is strongly suggestive that a Na^+–K^+-ATPase is involved in the potential responses. The insignificant (at 1% level) reduction of Q_{10} values ($1.22 \rightarrow 1.14$) by Ouabain in control solution probably reflects the reduced activity of the Na^+–K^+-ATPase in the absence of Na^+ substrate. Even in the presence of Ouabain, however, the Q_{10} values were still significantly greater than electrodiffusion theory allows. Other mechanisms which may be involved are a Na^+–Ca^{2+} exchange (Browning and Nelson, 1976; Connolly and Kerkut, 1983) or the various cotransport mechanisms in Tetrahymena which involve Na^+ such as Na^+ and sugars (Cirillo, 1962; Aomine, 1974). However the Na^+–Ca^{2+} exchange may be largely inactivated at resting potential so that this remaining Q_{10} difference is probably the sum of many small-scale processes.

The resting potentials of both Tetrahymena (Connolly and Kerkut, 1981) and Paramecium (Naitoh and Eckert, 1968) are strongly dependent upon external cations. The resting potential of Tetrahymena is depolarised by 28.3 mV for a 10-fold increase in $[K^+]_o$ compared to 30 mV in Paramecium. A 10-fold increase in $[Ca^{2+}]_o$ will lead to a 9.7 mV depolarisation in Tetrahymena and an 11 mV change in Paramecium. However these potential changes may not be the simple result of permeation of ions through the membrane. Protozoans have dilute intracellular fluid so that an influx of ions may lead to a change in tip potential which would contribute to the observed potential change (Satow, 1982). Eckert and Brehm (1979) discuss the possibility that increasing external cation concentrations results in surface charge neutralization and an increase in the potential difference across the membrane itself. When this is restored, presumably by conductance changes, the result is a depolarisation in the resting potential measured between the intracellular and extracellular bulk solutions (Kamada, 1934; Naitoh and Eckert, 1968; Connolly and Kerkut, 1981), an increase in input resistance and a positive shift in I-V relations (Satow and Kung, 1976; Eckert and Brehm, 1979; Satow, 1982). The rate of pumping of electrogenic transport mechanisms will also alter as the substrate concentration varies. Thus estimates of specific ion permeabilities based on electrophysiological data on bulk ion studies are liable to be inaccurate. Browning and Nelson (quoted in Machemer and DePeyer, 1977) have given an estimate

of $pK^+ : pNa^+ : pCa^{2+}$ of 1:0.1:0.1. However active transport of K^+ and Na^+ probably exaggerates the pK:pNa ratio, so that the correct ratio of permeabilities may be the order of magnitude of 1:1:0.1. Compared to other excitable cells the ciliates are generally much less permeable. Tetrahymena has a specific resistance of 3×10^4 Ωcm^2 whereas Aplysia neurone has a resistance of $2.2 \times 10^3 \Omega$ cm^2 (Tauc, 1955; Fessard and Tauc, 1956). Stoner and Dunham (1970) have shown that amino acids may be modified to maintain the osmotic balance between the internal and external environment, thus altering the Donnan equilibrium within the cell. Although $[CL^-]_i$ is maintained at a lower level than $[Cl^-]_o$ (Dunham and Child, 1961) CL^- is thought to contribute little to the resting potential in Paramecium (Naitoh and Eckert, 1974). Hence the major influences on resting potential would be $[Na^+]_o$, $[K^+]_o$ and the rate of pumping by electrogenic transport mechanisms.

An equation describing the steady state resting potential of Tetrahymena will probably be of the form (based on Moreton, 1969):

$$V = \frac{RT}{nF} \ln \frac{PK[K^+]_o + PNa[Na^+]_o + PCl[Cl^-]_i + M_T RT/FV}{PK[K^+]_i + PNa[Na]_i + PCl[Cl]_o + M_T RT/FV}$$

where

$$M_T = M_K + M_{Na^+} + M_{Ca^{2+}} - M_{Cl^-}$$

and M is the ion flux across the membranes.

This equation assumes that H^+, OH^- and PO_4^{3-} make little contribution to the resting potential and that the permeabilities of Mg^{2+} and Ca^{2+} are very small compared to K^+ and Na^+. From these studies it would seem that the electrogenic pump term, $M_T RT/FV$, may account for up to 30% of the membrane potential. More work involving changes of $[K^+]_o$ in the presence and absence of Na^+, Ouabain and cold is necessary to evaluate the $pK^+ : pNa^+$ ratio accurately. However, as noted above, whatever technique is used to estimate ion permeabilities tends to affect the value obtained. Estimating ion permeabilities is further complicated by the physical structure of Tetrahymena. The somatic membrane is underlain by alveolar sacs which make it effectively a 3-layered structure (the pellicle). The sacs may also provide a buffered zone between the bulk of the intracellular fluid and the external solution. The alveoli do not extend into the intraciliary space, so that the permeability of the ciliary membrane may be greater than that of the pellicle. Furthermore the alveoli and basal plate compartment the cilia from the cell to some extent and this could lead to a partial screening of electrical events in the cilia.

Yamaguchi (1960) noted that potential oscillations with an amplitude of 18 mV were observed when the recording electrode was placed near the anterior contractile vacuole of Paramecium. Similar oscillations have been occasionally recorded in Tetrahymena (Connolly and Kerkut, 1981) and Zoothamnium (Moreton and Amos, 1979). The fact that such a large voltage change was not seen when recording distally to the CV suggests that the cell is not isopotential. However the inference drawn from measurements made with two recording electrodes placed in the cell soma was that the bulk of the somatic intracellular fluid is isopotential (Eckert and Naitoh, 1970). This implies that these cells may screen electrical activity in localised areas by separating these systems from the bulk solution by membranes of high resistance. Such an arrangement has been proposed for the ciliary plaques (Connolly and Kerkut, 1983), which may screen $Na^+ : Ca^{2+}$ exchange. Biochemical investigations in both Tetrahymena (Nozawa and Thompson, 1971) and Paramecium (Kaneshiro *et al.*, 1979) have demonstrated that membranes from functionally and anatomically different parts of the cell differ in composition and therefore may differ in permeability. Furthermore in Paramecium voltage sensitive Ca^{2+} channels are known to be located in the cilia (Ogura and Takahashi, 1976; Dunlap, 1977) and there is assymmetric distribution of mechanoreceptive channels in both Tetrahymena (Connolly and Kerkut, 1981) and Paramecium (Naitoh and Eckert, 1972). If specialised membranes do produce electrical heterogeneity and screening in Tetrahymena, then the same phenomena may occur in more advanced excitable cells such as mammalian pyramidal cells. The electrical membrane constants derived for excitable cells should therefore be regarded as averages for the many different areas of membrane within the cell such as the axon hillock and post synaptic membranes as well as the cell soma.

The hyperpolarising effect of an electrogenic Na^+–K^+ (Mg^{2+})-ATPase may have an anti-arrhythmic effect in Tetrahymena as exists in the heart (Gadsby and Cranefield, 1979) and it would help to prevent the many fluctuations in membrane potential caused by such processes as CV pumping from triggering regenerative depolarisations.

In conclusion, the evidence presented here suggests that there is an electrogenic contribution of up to 30% to the membrane potential in Tetrahymena which may be mainly the result of ion separation by a Ouabain sensitive Na^+–K^+ (Mg^{2+})-ATPase.

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RESTING METABOLIC RATE AND ENERGETIC COST OF INCUBATION IN MACARONI PENGUINS (*EUDYPTES CHRYSOLOPHUS*) AND ROCKHOPPER PENGUINS (*E. CHRYSOCOME*)

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Abstract – 1. Oxygen consumption of resting and incubating Macaroni Penguins (*Eudyptes chrysolophus*) and Rockhopper Penguins (*E. chrysocome*) was measured at Marion Island in the sub-Antarctic.

2. Resting metabolic rate of Macaroni Penguins of mean mass 3779 g averaged 1160.7 kJ/day and that of Rockhopper Penguins of mean mass 2506 g averaged 862.8 kJ/day.

3. Incubating metabolic rates were 1031.8 kJ/day for Macaroni Penguins of mean mass 4839 g and 7014 kJ/day for Rockhopper Penguins of mean mass 2767 g.

4. Energetic cost of incubation was significantly lower than resting metabolic rate in both species but was close to predicted values of basal metabolic rate for birds of equivalent masses.

5. Incubation may not be energetically expensive for small penguins, and incubating metabolic rate may represent a better indication of basal metabolic rate than does resting metabolic rate.

INTRODUCTION

Penguins are distributed from the Antarctic to the tropics. Their semi-aquatic lifestyles, reported low body temperatures and long fasts during breeding and moulting have resulted in considerable interest in their thermoregulation and metabolic rates in air and water (Kooyman *et al.*, 1976; Stahel and Nicol, 1982), as well as during prolonged fasts (Le Maho and Despin, 1976; Le Maho *et al.*, 1976; Pinshow *et al.*, 1976). Estimates of metabolic rate, based on oxygen consumption measurements, are available for several species: in particular, basal metabolic rate (BMR) and standard metabolic rate (SMR) have been measured for Macaroni Penguins (*Eudyptes chrysolophus*) and Rockhopper Penguins (*E. chrysocome*) (Gavrilov, 1977). Information on incubating penguins, however, has been confined to estimates based on rates of mass loss during the incubation fast. Such estimates require some knowledge of the composition of the metabolites oxidized for energy production during the fast.

The purpose of this study was to measure metabolic rate and energetic cost of incubation in Macaroni Penguins and Rockhopper Penguins and to compare these with metabolic rates of other penguins, and with estimates of incubation costs based on rates of mass loss.

MATERIALS AND METHODS

The study was carried out at sub-Antarctic Marion Island (46°52′S, 37°51′E) between December 1981 and April 1982.

Resting metabolic rate

RMRs were measured on five Macaroni Penguins (three males and two females) and on four Rockhopper Penguins (two males and two females). All birds used for RMR measurements were feeding small chicks and so were not undergoing lengthy, natural fasts.

Oxygen consumption ($\dot{V}_{O_2}$) was measured in the laboratory in a translucent, airtight metabolic chamber (400 mm dia × 750 mm high) using an open, flow-through system. Air, drawn from outside the laboratory, was pumped through a regulating flow meter before entering the chamber. Air exiting the chamber was passed through a silica gel drying tube, a rotameter flowmeter and a silica gel/carbosorb tube before entering a Taylor-Servomex OA 570 paramagnetic oxygen analyzer. Flow rate was set to between 2500 and 3500 ml/min. This was sufficient to produce a drop in oxygen content in the expired air of between 1 and 2% below that of ambient air. The oxygen analyzer was calibrated with nitrogen before the experiment and the oxygen content of ambient air was checked at regular intervals throughout the run. A thermocouple, inserted into the chamber through a rubber bung, measured chamber temperature. An initial period of at least 1 hr was allowed for the birds to settle and the chamber air to equilibrate before the first reading was taken. Thereafter, readings of chamber temperature, flow rate and the percentage of oxygen in the expired air were recorded at 30 min intervals over 24 hr at normal photoperiod. Chamber temperature during RMR measurements ranged from 11.6 to 15.9°C (mean = 13.4°C).

Incubating metabolic rate

Incubating adult birds and their eggs were removed from the nest and placed in the chamber in the laboratory. A shallow metal tray filled with stones and gravel prevented the eggs from rolling about in the chamber. Five Macaroni and five Rockhopper Penguins incubated readily in the chamber. A further two birds of each species covered their eggs but did not adopt the normal incubation posture. These were regarded as not incubating and the results were not used in later calculation of incubating metabolic rate (IMR). Oxygen consumption was measured as already described for RMR. Temperature range in the chamber during IMR measurements was 8.7–17.5°C (mean = 13.5°C).

$\dot{V}_{O_2}$ (STPD) for both RMR and IMR were calculated from the lowest, stable period during the respective 24 hr runs, using the equation of Hill (1972). Stable periods ranged from 1 to 9 hr and 4 to 12 hr for measurements of RMR and IMR

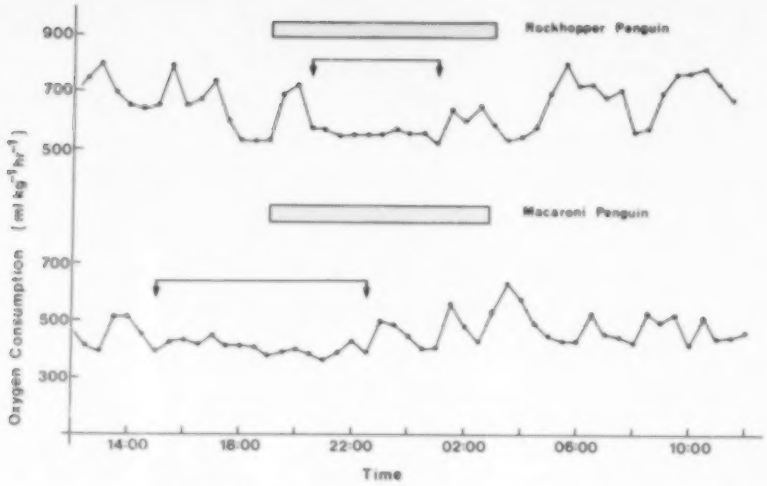

Fig. 1. Oxygen consumption for an incubating Rockhopper and Macaroni Penguin over 24 hr at normal photoperiod. Dotted areas indicate hours of darkness and arrows indicate the stable periods from which IMR was calculated.

respectively. $\dot{V}_{O_2}$ was converted to an energy equivalent using 1 l O_2 = 20.084 kJ. Error estimates quoted are ±1 SD.

RESULTS

Examples of $\dot{V}_{O_2}$ for a Macaroni and a Rockhopper Penguin over 24 hr at normal photoperiod, showing the stable periods from which IMR was calculated, are illustrated in Fig. 1. RMR was determined similarly.

RMRs and IMRs of Macaroni and Rockhopper Penguins are presented in Table 1. Included are the mean metabolic rates measured over 24 hr and the predicted BMR from equation (5.5) of Kendeigh *et al.* (1977).

Mean metabolic rates over 24 hr, here referred to as the average daily metabolic rate (ADMR) and the average daily incubating metabolic rate (ADIMR), were between 16 and 20% greater than RMR and IMR calculated from the stable periods. Measured RMR from stable periods was 25 and 26% greater than predicted BMR for Macaroni and Rockhopper Penguins respectively. Measured IMR was close to predicted BMR, but significantly lower in both species than the measured RMR when compared on a mass specific basis (Macaroni, $t = 15.62$, $P < 0.001$, $df = 135$; Rockhopper, $t = 20.79$, $P < 0.001$, $df = 110$).

DISCUSSION

BMR is regarded as the lowest level of metabolism of an organism during normal existence, upon which the energy cost of all activities is superimposed

Table 1. Resting metabolic rate (RMR), incubating metabolic rate (IMR) and average daily metabolic rates during rest (ADMR) and incubation (ADIMR) for Macaroni and Rockhopper Penguins, with values for predicted BMR for comparison

Resting Metabolic Rate

	Mean mass (g)	No. of birds	RMR (kJ day^{-1})	RMR (kJ g^{-1} day^{-1})	ADMR (kJ day^{-1})	BMR[a] (kJ day^{-1})	RMR/BMR
Macaroni Penguin	3 779	5	1 160.7 ± 148.9	0.307 ± 0.039	1 319.6 ± 197.4	929	1.25
Rockhopper Penguin	2 506	4	862.8 ± 163.0	0.344 ± 0.065	1 001.3 ± 172.8	687	1.26

Incubating Metabolic Rate

	Mean mass (g)	No. of birds	IMR (kJ day^{-1})	IMR (kJ g^{-1} day^{-1})	ADIMR (kJ day^{-1})	BMR[a] (kJ day^{-1})	IMR/BMR	IMR/RMR
Macaroni Penguin	4 839	5	1 031.8 ± 250.8	0.213 ± 0.052	1 242.9 ± 262.3	1 114	0.93	0.69
Rockhopper Penguin	2 767	5	701.4 ± 36.7	0.253 ± 0.013	837.8 ± 210.9	739	0.95	0.74

[a] Predicted from Kendeigh *et al.* (1977).

(Kendeigh *et al.*, 1977). As defined by Kendeigh *et al.* (1977), it is measured on an organism which is in a resting, postabsorptive state within its thermoneutral zone.

Birds used in this study were caught when returning to sea after feeding chicks and were considered to be postabsorptive. Chamber temperatures during the study were within the range that the birds might normally experience at Marion Island during summer (11.6–15.9°C, mean = 13.4°C). Gavrilov (1977) calculated lower critical temperatures of Macaroni, Rockhopper and King Penguins (*Aptenodytes patagonicus*) to be 12, 16 and 3°C respectively, but Le Maho (1983) reports a lower critical temperature of −5°C for the King Penguin. Since Macaroni, Rockhopper and King Penguins are found sympatrically throughout many parts of their range, it is likely that the lower critical temperatures reported by Gavrilov (1977) for the Macaroni and Rockhopper Penguins are also overestimates, and that the birds in this study were within their thermoneutral zones. This is supported by the observation that there was no significant correlation between temperature and mass specific metabolic rate in Macaroni and Rockhopper Penguins during measurement of RMR over the narrow temperature range of the measurements ($r = -0.151$, $P > 0.1$). The metabolic rate of incubating birds, however, was significantly lower than that of non-incubating birds measured under similar conditions. Consequently, although conditions comply with the definition of BMR *sensu* Kendeigh *et al.* (1977), the metabolic rate measured cannot be regarded as a measure of true BMR in the traditional sense. For this reason, for the purpose of this study, measured metabolic rate of non-incubating penguins resting in a postabsorptive state, probably within their thermoneutral zones, is referred to as RMR.

The question arises as to how many other measurements of metabolic rate, referred to as BMR, are in fact true estimates of BMR. The need for workers to describe the conditions under which the measurements were made, and to define the terms which they use to describe these measurements, is clearly highlighted.

Resting metabolic rate

RMRs of Macaroni and Rockhopper Penguins measured in this study were higher than the predicted BMR for birds of equivalent mass. Gavrilov (1977) measured BMR and SMR (metabolic rate below the zone of thermoneutrality) on zoo specimens of Macaroni and Rockhopper Penguins. He found that BMR of Macaroni Penguins of mean mass 3870 g was 747 kJ/day while that of Rockhopper Penguins of mean mass 2330 g was 504 kJ/day. However, the figures presented in the text by Gavrilov (1977) for Macaroni Penguins do not agree with those presented in his tables, the former being 1035.1 kJ/day as opposed to the figure quoted above. Data for energy expenditure from Gavrilov (1977) quoted in this paper were taken from the tables. These are 21 and 23% lower respectively than the predicted BMR from the equation of Kendeigh *et al.* (1977), and 46 and 49% lower respectively than the RMR of Macaroni and Rockhopper Penguins measured in this study. SMRs measured by Gavrilov (1977) on the same birds were 1275 and 764 kJ/day for Macaroni and Rockhopper Penguins respectively. Although Gavrilov does not give the temperatures at which SMR measurements were made, from data presented in his Fig. 1 it is estimated that temperatures were 0°C for Macaroni Penguins and 5°C for Rockhopper Penguins. Gavrilov's figures for SMR are more comparable to the RMRs measured in this study, differing by 7% and 4% respectively.

Estimates of metabolic rates in Macaroni Penguins also have been made by Scholander (quoted in Weathers, 1979). These have been discounted here because they were measured on forcibly submerged, struggling birds, a condition certain to result in elevated metabolic rates.

Available data on metabolic rates in other non-incubating penguins, derived from oxygen consumption measurements, are summarized in Table 2. They can be compared with those for RMR of Macaroni and Rockhopper Penguins using the ratios of the measured metabolic rates to the predicted BMR for birds of equivalent mass. RMRs of both Macaroni and Rockhopper Penguins are comparable with metabolic rates of other species although it is evident that there is considerable variation in measured metabolic rates, both between and within species. Some of this variation may be accounted for by differences in experimental techniques, the physiological state of the experimental birds and the conditions under which oxygen consumption was measured. It is noteworthy that measurements of metabolic rate on zoo penguins (i.e. Drent and Stonehouse, 1970; Gavrilov, 1977) are the lowest reported and all are lower than predicted BMR. Whether this is due to acclimatization of the birds to ambient conditions at their respective locations, familiarization of the birds to handling and disturbance, or both, is not certain.

Penguins are reported to have lower body temperatures than other groups of birds (McNab, 1966; Dawson and Hudson, 1970), a feature that Warham (1971) has suggested implies low metabolic rates. Although it now appears that low body temperatures in penguins may be due to site of measurement (Goldsmith and Sladen, 1961; Drent and Stonehouse, 1971; Le Maho *et al.*, 1976; Stahel and Nicol, 1982), the hypothesis of Warham (1971) is nevertheless supported if the metabolic rates of zoo penguins are regarded as being more representative of BMR than those of wild birds in temporary captivity. However, the mean overall level of metabolism of wild penguins in temporary captivity (excluding SMR measurements of Gavrilov (1977)) is 16% greater than predicted. Elevated levels of metabolism have also been observed in aquatic and semi-aquatic mammals (Irving, 1973; Morrison *et al.*, 1974) and have been explained as an adaptation to maintain body temperature in water which has a higher cooling capacity than air. The variation in measured metabolic rates precludes resolution of these hypotheses until further, more standardized, information on penguin metabolism becomes available.

The regression of log metabolic rate against log body mass for the data presented in Table 2 is illustrated in Fig. 2. The regression is described by the equation $M = 2.32\ W^{0.74 \pm 1.01}$ ($r = 0.97$), where M is the metabolic rate in kJ/bird per day and W is the body mass in grams. The slope of the regression line

Table 2. Metabolic rates of penguins, derived from oxygen consumption measurements (data from the literature)

Species	No.	No. of birds	Mean mass (g)	Metabolic Rate measured	Metabolic Rate (kJ day^{-1})	Metabolic Rate (kJ kg^{-1} day^{-1})	Predicted BMR[a] (kJ day^{-1})	MR/BMR	Source
Emperor Penguin	1	4	31 750	RMR(fasting)	5 791[b]	182	4 436	1.31	Dewasmes et al. (1980)
Aptenodytes forsteri	2	11	24 800	BMR(fasting)	4 239	171	3 700	1.15	Le Maho et al. (1976)
	3	5	23 370	SMR(fasting)	3 704	159	3 542	1.04	Pinshow et al. (1976)
	4	4	20 790	RMR	3 307	159	3 250	1.02	Pinshow et al. (1977)
King Penguin	5	7	13 270	BMR	2 237	169	2 337	0.96	N.J.Adams (unpubl.data)
A. patagonicus	6	3	12 662	RMR(fasting)	2 877[c]	227	2 258	1.27	Le Maho & Deepln (1976)
	7	8	11 080	BMR	1 888	170	2 047	0.92	Gavrilov (1977)
				SMR	2 099	189	2 047	1.02	
Gentoo Penguin	8	4	6 290	RMR	1 605	255	1 350	1.19	N.J.Adams & C.R.Brown
Pygoscelis papua									(unpubl.data)
Yelloweyed Penguin	9	1	4 800	BMR	996	208	1 107	0.90	Stonehouse (1968) In
Megadyptes antipodes									Drent & Stonehouse (1971
Adelie Penguin	10	-	ca 4 500[d]	BMR	1 562	347	1 056	1.48	Le Resche & Boyd (1969)
Pygoscelis adeliae	11	14	3 970	RMR	1 057	266	963	1.10	Kooyman et al. (1976)
Peruvian Penguin	12	3	3 870	BMR	820	212	945	0.87	Drent & Stonehouse (1971
Spheniscus humboldti									
Macaroni Penguin	13	4	3 870	BMR	747	193	945	0.79	Gavrilov (1977)
Eudyptes chrysolophus				SMR	1 275	330	945	1.35	
	14	5	3 780	RMR	1 161	307	929	1.25	This study
Fjordland Penguin	15	4	2 600	BMR	598	230	703	0.85	Stonehouse (1968) In
E. pachyrhynchus									Drent & Stonehouse (1971
Rockhopper Penguin	16	4	2 506	RMR	863	344	687	1.26	This study
E. chrysocome	17	2	2 330	BMR	504	216	651	0.77	Gavrilov (1977)
				SMR	764	328	651	1.17	
Whiteflippered Penguin	18	3	1 150	RMR	571	497	388	1.47	Pinshow et al.(1977)
Eudyptula albosignata									
Little Penguin	19	6	900	BMR	383	422	324	1.18	Stahel & Nicol (1982)
Eudyptula minor									

[a] From Kendeigh *et al.* (1977); BMR = 0.5224 $W^{0.7347}$ where BMR is in kcal/day, converted here to kJ/day, and W is the body mass in grams.
[b] Using the relationship between body mass and metabolic rate where $y = 1.238x + 27.72$, where x is the body mass in kg and y is the metabolic rate in Watts, converted to kJ/day, calculated from text-figure points at the midpoint of the fast.
[c] Using the relationship between body mass and metabolic rate from Croxall (1982) where $y = 0.0043x - 21.0$; x is the body mass in grams and y is the metabolic rate in Watts, converted to kJ/day, calculated from text figure points after day 5.
[d] Mass from Croxall (1982).

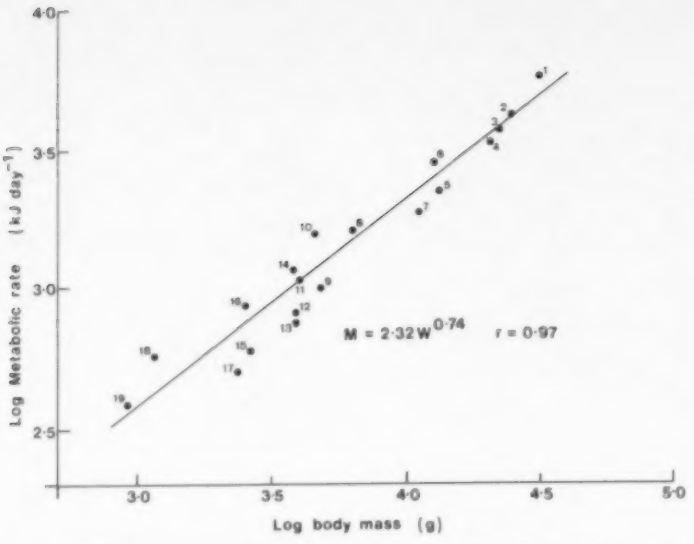

Fig. 2. Relationship between log metabolic rate, based on oxygen consumption measurements, and log body mass in penguins, from data presented in Table 2. Numbers correspond to those in Table 2 and the SMR measurements of Gavrilov (1977) are excluded.

(0.74) is identical to that obtained by Croxall (1982) for only ten sets of measurements on seven species of penguin and very close to the slope of 0.73 derived by Kendeigh *et al.* (1977) for non-passerines. This supports the suggestion of Croxall (1982) that penguins have similar body mass/metabolism relationships to other birds.

Incubating metabolic rate

Energy expenditure of incubating Macaroni and Rockhopper Penguins was significantly lower than RMR of non-incubating birds when compared on a mass specific basis, but was close to predicted BMR in both species. This result is surprising, and suggests that there may be some extrinsic factor resulting in elevated rates of metabolism in non-incubating birds. Incubating birds in the laboratory were noticeably more restful than non-incubating birds, and the stable periods from which IMR was calculated generally were longer than those used to calculate RMR, although the difference only approaches significance ($t = 2.104$, $0.1 > P > 0.05$). Comparison of data for King, Gentoo (*Pygoscelis papua*) and Adélie (*P. adeliae*) Penguins led Croxall (1982) to suggest that stress in fasting, non-incubating birds might result in a greater energy expenditure than that required by incubating birds for egg temperature maintenance. It is probable that handling and disturbance during the chick-rearing period might represent a similarly greater stress than that imposed on incubating birds. It is notable that measurements on pre-moult Macaroni and Rockhopper Penguins have RMRs similar to those measured in this study (C. R. Brown, unpublished data). Consequently, for Macaroni and Rockhopper Penguins, IMR taken from the lowest stable periods might represent a closer estimate of BMR than that measured on non-incubating birds. A further factor which possibly needs to be taken into account when comparing mass specific IMR and RMR is the excess mass of incubating birds relative to birds feeding chicks (Table 1). A large proportion of this excess mass comprises stored fat which is metabolically inert. This in itself would tend to result in a proportionately lower mass specific metabolic rate for incubating birds when compared to those feeding chicks.

No estimates for the energy cost of incubation in Rockhopper Penguins are available for comparison with the results of this study, but estimates of the energy cost of incubation in Macaroni Penguins, based on rates of mass loss in non-captive, incubating birds, have been made by Carrick (1972) and by Croxall (1982). In order to estimate energy expenditure from rates of mass loss, some knowledge of the composition of metabolites oxidized during energy production is required. For penguins, figures derived by Groscolas and Clément (1976) from incubating Emperor Penguins (*Aptenodytes forsteri*) are generally used. They found mass loss to comprise 55.5% fat, 9.2% protein and 35.3% water. Since estimates based on rates of mass loss represent average daily metabolic rates, they are compared here with the metabolic rate of incubating birds measured over 24 hr in the chamber (ADIMR) rather than with IMR calculated from the lowest stable period. Mean ADIMR of Macaroni Penguins, measured in the present study was 1.12 times the predicted BMR. This is considerably lower than estimates of 1.50 and 1.91 times predicted BMR obtained from mass loss data of Carrick (1972) for two birds, but compares well with those of Croxall (1982), who obtained figures between 1.17 and 1.25 times predicted BMR. The similarity in the estimates of Croxall (1982) with those of this study, suggests that the composition of metabolites oxidized, derived by Groscolas and Clément (1976), are realistic for penguins.

The mean daily energy cost of incubation in penguins, estimated by Croxall (1982), is 1.39 times predicted BMR (range 1.02–1.57). ADIMR of both Macaroni and Rockhopper Penguins are within this

range and daily incubation costs of penguins 10–30% greater than BMR appear reasonable.

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EFFECT OF DIFFERENT FACTORS ON THE METABOLISM OF *MORIMUS FUNEREUS* LARVAE

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Abstract—1. The complex effect of different factors such as, for the species unfavourable constant temperature (23°C, 7 days), deprivation of food (4 days at 23°C), injection of potassium saline and/or cerebral-complex extract into larvae of *Morimus funereus* on the activity of the digestive enzymes (protease, amylase) and haemolymph trehalose concentration has been studied.

2. The parameters studied are listed in decreasing order of susceptibility to the factors studied: protease, amylase, trehalose.

3. The results are discussed from the aspect of the role of neurohormones in the regulation of selected biochemical parameters and from the aspect of protective adaptive mechanisms.

INTRODUCTION

A knowledge of animal integrative and regulatory systems whose functional unit is the neuron (conventional and neurosecretory) is a prerequisite for understanding of the adaptive processes and the evolution (Scharrer, 1978; Maddrell and Nordmann, 1979; Polenov, 1980). Some recent work on neurosecretion and neurohormones has revealed the multiple significance of the neurohormones in the metabolism of insects. (Steele, 1976; Raabe, 1982; Steel, 1982).

The results of our previous investigations have suggested that in the larvae of phytophagous Coleoptera living permanently in the food medium (*Tenebrio molitor* and *Morimus funereus*), the midgut proteolytic (SPA) and amylolytic (SAA) activities are controlled by neurohormones (Ivanović *et al.*, 1978). Evidence was provided that in some other insect species the haemolymph trehalose level is regulated by neurohormones as well (Friedman, 1978; Kramer, 1980).

The aim of the present work was to broaden the scope of our study on the role of neurohormones in the regulation of digestive enzyme activity and haemolymph trehalose concentration in the larvae of *Morimus funereus* under the complex effect of external and internal factors (for the species unfavourable constant temperature, deprivation of food, injection of potassium saline and/or cerebral-complex extract into larvae). It was expected that the comparative investigation of the above biochemical parameters might provide more knowledge about the physiological role of neurohormones in the insect metabolism. In addition, it was supposed to find out under which conditions the experimental factors were favourable, unfavourable or stressory for the organism.

MATERIALS AND METHODS

Insects, treatments

Larvae of the cerambycid beetle *M. funereus* collected from oak stumps of a saw-mill depot (Morović) at the end of September were used for experiments immediately after being transferred to the laboratory. The control larvae from the natural habitat (NC) were killed at the beginning of the experiment and their midguts and haemolymph used for the study of the digestive enzyme activity and trehalose concentration, respectively. Isolated larvae fed with minced oak bark and divided into identical experimental groups according to their body wt (300–1000 mg) were exposed to 23°C during 7 days. One group of larvae was then killed (F) while the remainder of them were starved at the same temperature (ST, 23°C + ST). After 4-day starvation the controls were killed, and the other starved larvae were injected with 0.7% KCl solution (S) and/or cerebral-complex extract (E). The extract was prepared from the larval cerebral-complex (without corpora allata) homogenate suspended in potassium saline (18 heads/ml), and filtered prior to injection. The donors of the cerebral-complex were larvae (body wt 100–200 mg) maintained at natural conditions, with high digestive enzyme activity. Each recipient received 0.2 μl/mg body wt of the extract or potassium saline. The treated larvae were killed at different time intervals following injection (see Figs 1, 2 and 3) but always between 7 and 9 a.m.

Proteolytic and amylolytic activities (SPA, SAA)

Proteolytic and amylolytic activities were determined in aqueous midgut extracts (each midgut separately) as reported in a previous paper (Ivanović *et al.*, 1975a). Three replicate tests were performed for each set of experimental conditions. The enzyme activities were expressed in per cent of sp. act., the controls NC and/or 23°C + ST taken as 100%.

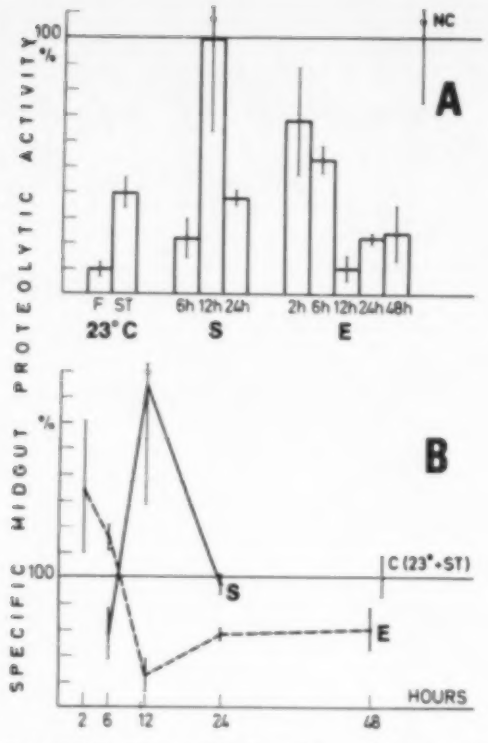
Determination of the trehalose concentration

The concentration of trehalose was determined in pooled haemolymph spectrophotometrically at 630 nm using the anthron reaction (Wyatt and Kalf, 1957). The trehalose was precipitated with 10% TCA and centrifugated at 5500 revs/min. The concentration was calculated from the standard curve of trehalose and presented as per cent, taking the controls as 100%. Two repetitions were made for each set of experimental conditions.

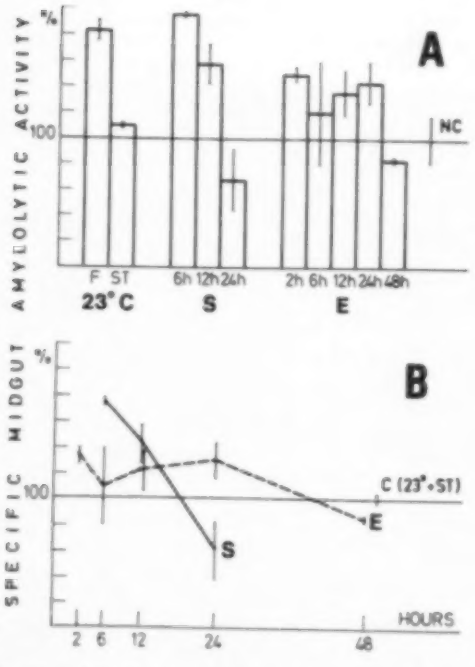
RESULTS

Changes in proteolytic activity (SPA)

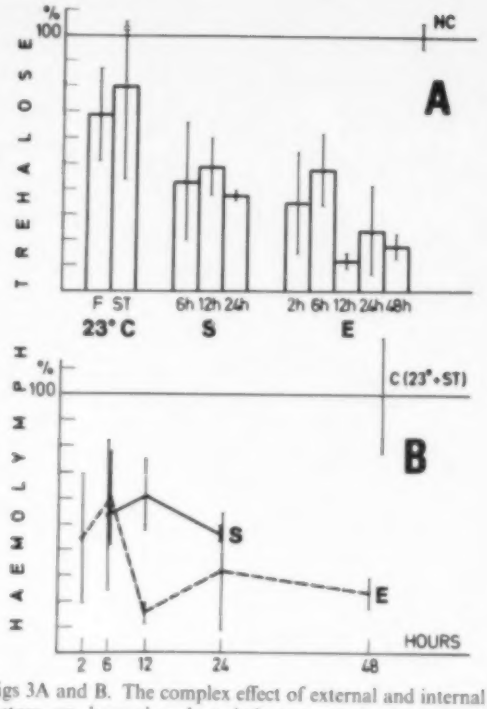
The results of the study concerned with changes in midgut proteolytic activity of *M. funereus* larvae under different experimental conditions are presented in



Figs 1A and B. The complex effect of external and internal factors on midgut proteolytic activity of *M. funereus* larvae. C(23°C + ST)—control, acclimation at 23°C and starvation; E—cerebral-complex extract; F—fed larvae; NC—control, natural conditions; S—KCl saline; ST—starved larvae.



Figs 2A and B. The complex effect of external and internal factors on midgut amylolytic activity of *M. funereus* larvae. Symbols as in Fig. 1.



Figs 3A and B. The complex effect of external and internal factors on haemolymph trehalose concentration of *M. funereus* larvae. Symbols as in Fig. 1.

Figs 1A and B. It is evident that except for one experimental series, all the performed treatments exhibited significant decrease of SPA of the experimental larvae, ranging from 10 to 70% in relation to the control (NC). Only in the larvae injected with saline solution SPA reaches the level of the control 12 h after treatment. A 7-day exposure of fed larvae to 23°C results in a decline of SPA to 10%, while the subsequent 4-day starvation at the same temperature increases SPA of larvae by 30% (compensatory reaction) when compared with that of the fed ones. The injection of potassium saline and/or cerebral-complex extract into starved larvae held at 23°C elicits at the level of SPA different larval responses at various time intervals after treatment (Fig. 1B). Potassium saline provokes 6 h after injection a decline, and 12 h after treatment on abrupt rise of SPA (250%). A decrease of SPA to the control level is noted 24 h after saline injection. In larvae injected with the extract, 2 h later there is a significant increase of SPA (168%) followed by its gradual decrease to 25% (12 h). Again a rise of SPA to about 50% was noted (24 h) and it is maintained at the same level until the end of the experiment (48 h).

Changes in amylolytic activity (SAA)

Changes in midgut amylolytic activity of *M. funereus* larvae under the effect of different treatments are shown in Figs 2A and B. Figure 2A indicates that in the majority of the experimental larvae SAA is increased. This increase ranges from 10 to 100% when compared with the enzyme activity of the controls (NC). The temperature of 23°C rises significantly SAA of fed larvae in contrast to its effect on SPA (Fig. 1A). Starvation, which evokes at the level of SPA a compensatory reaction of the larvae, decreases SAA.

The maximum level of SAA is reached in saline-injected larvae 6 h after treatment (Fig. 2B). It is interesting to note that under identical experimental conditions SPA was decreased. Prolonged exposure of the larvae to the effect of saline at 23°C resulted in a gradual fall of SAA, dropping 24 h after injection below the control value. The extract-injection evokes a rise of SAA in comparison to the controls, with the maximum 24 h after treatment. Here, the increase of SAA amounts about 30% compared with the control (23°C + ST), but 100% when compared with the saline control. In the course of the experiment the effect of the extract on SAA decreases, i.e. 48 h after injection SAA falls beneath the level of the control.

Changes in trehalose concentration

The results on changes of the trehalose level in the haemolymph of *M. funereus* larvae are presented in Figs 3A and B. In all the treated larvae there is a decrease in the concentration of the haemolymph trehalose in comparison to the controls (NC). The most remarkable decrease is obtained under the influence of the cerebral-complex extract, the lowest one by the effect of the external factors (23°C, lack of food). Under natural conditions the concentration of haemolymph trehalose show little individual variability, whereas unfavourable external factors increase it considerably. It is evident from Fig. 3B that potassium saline decreases the amount of trehalose below the level of the control (23°C + ST), and that its effect does not provoke significant changes in the haemolymph trehalose concentration in the course of the experiment, as stated for SPA. Compared with the saline control, the injected extract does not affect the level of trehalose 2–6 h after treatment, but prolonged exposure of the larvae to its effect (12–48 h) results in a decrease of the trehalose concentration. The lowest level was observed 12 h after the extract injection.

DISCUSSION

The complex effect of the experimental factors (temperature, starvation, injection of potassium saline and cerebral-complex extract into larvae) evokes significant changes in the activity of the digestive enzymes and the level of haemolymph trehalose. Evidence was provided that in several insect species the biochemical parameters studied in this work are controlled by neurohormones (Maddrell and Nordmann, 1979; Raabe, 1982). Moreover, it was suggested that midgut SAA and SPA of two phytophagous species of Coleoptera, *Tenebrio molitor* and *Morimus funereus* are regulated by neurohormones (Dadd, 1961; Mordue, 1967; Janković-Hladni *et al.*, 1978; Ivanović *et al.*, 1978). Hence, the obtained results will be discussed from the aspect of the regulatory role of neurohormones in insect metabolism.

Temperature

Exposure of *M. funereus* larvae to a constant temperature, unfavourable for the species has provoked a drastic fall of SPA and a considerable rise of SAA, whereas the concentration of haemolymph trehalose was only a little lowered. The response of the larvae was more rapid at the level of the digestive enzymes involved in protein and carbohydrate metabolism than at the level of the haemolymph trehalose. It was therefore concluded that the first mentioned parameters are suitable as the criteria for the evaluation of the intensity of a stress. The concentration of haemolymph trehalose, a component of the internal environment, show changes of low intensity. It has been previously reported that prolonged exposure of the larvae to 23°C (15 days) decreases the activity of both digestive enzymes (Ivanović *et al.*, 1978). The results of recent experiments obtained after a week of larval acclimation at 23°C agree with those reported in a previous paper using winter larvae of *M. funereus* in experiments (first critical period). When larvae collected in other seasons of the year were used in experiments, only results concerning SPA were in agreement with the recent ones (Ivanović *et al.*, 1979; Ivanović *et al.*, 1982; Janković-Hladni *et al.*, 1980). It was suggested that stress conditions, dependent upon their duration, switch on in the larvae protective mechanisms, i.e. a compensatory response at the level of digestive enzymes involved in protein and carbohydrate metabolism (Janković-Hladni *et al.*, 1980).

The decline in the trehalose level in the haemolymph of *M. funereus* larvae exposed to 23°C is similar to that observed in the diapause pupae of *Philosamia cynthia* exposed to a temperature of 20–25°C (Hayakawa and Chino, 1982). In insects treated with high temperatures the drop in trehalose conc. might be explained by the inactivation of glycogen phosphorylase and the activation of glycogen synthetase, the enzymes responsible for the interconversion between glycogen and trehalose. It appears that in *Phormia*, the hypotrehalosemic hormone (Chen and Friedman, 1977a,b) does not affect the fat body glycogen synthetase, but that it induces a decrease in UDP glucose:glucose phosphate glucosyltransferase activity (Raabe, 1982). By which mechanism the decrease in the haemolymph trehalose level in *M. funereus* larvae is brought about is not yet clear. It is noteworthy that larvae of *M. funereus* exposed to low temperatures (−1°C) show an increase in the amount of haemolymph trehalose.

The comparative investigations of the neuroendocrine system and the activity of digestive enzymes of *M. funereus* larvae have shown that the peptidergic neurons type A (medial group in the protocerebrum and two medioventral neurons in the suboesophageal ganglion) respond to the effect of an unfavourable temperature (23°C) by depressing their activity, reflected in decreased release and synthesis of the neurosecretory material (Ivanović *et al.*, 1975a,b).

Starvation

As reported for several insect species not allowed to feed, also in starved larvae of *M. funereus* SPA and SAA were significantly decreased (Ivanović *et al.*, 1978). Our results indicate that under the complex effect of starvation and an unfavourable temperature, a significant rise in SPA was attained (compensatory reaction). It should be noted that in temperature-treated fed larvae SAA shows a compensatory reaction, while in the starved ones it decreases. It was shown that deprivation of food slows down the release of the neurosecretory material from A1 neurosecretory cells of *Bombyx mori* larvae (Panov, 1967) and *Tenebrio molitor* adults (Janković-Hladni, 1971). One can conclude therefore, that the increased SPA is the con-

sequence of the increased release of the neurosecretory material, induced through a double stress. Ivanović *et al.* (1980) have reported that the response of *M. funereus* larvae to experimental factors is dependent, at any level of the organization, on the previous physiological state of the larval cerebral neurosecretory cells. There is yet no answer to the question raised: which mechanisms are switched on during larval starvation under natural conditions to affect the decrease of digestive enzyme activity and which ones during starvation at an unfavourable temperature (double stress) to induce a rise of SPA.

In starved larvae of *M. funereus* the level of haemolymph trehalose remained substantially unchanged. The obtained S.E. values suggest that in some individuals this parameter was also increased. Terra and Ferreira (1981) have demonstrated that in starved larvae of *Rhynchosciara americana* the activity of the digestive enzymes (amylase, cellulase, trehalase, aminopeptidase, trypsin) was decreased, whereas the haemolymph trehalose concentration remained unaffected by starvation.

Potassium saline

It was found that potassium saline affect considerably all the biochemical parameters examined. The activity of digestive enzymes was dependent on the exposure time of the larvae to the effect of saline. Independently of the exposure time, the amount of haemolymph trehalose declined. Having in view that the parameters studied were reported to be regulated by neurohormones, the remarkable effect of potassium saline is probably due to the effect of K ions on the membrane depolarization, causing in animals the release of neurohormones (Maddrell and Nordmann, 1979). Several authors have demonstrated that in various insect species solutions with high K^+ conc. and in the presence of Ca^{2+} induce the release of different neurohormones (Gersch *et al.*, 1970; Maddrell and Gee, 1974; Girardie and Girardie, 1977; Orchard *et al.*, 1981). The question was raised: for what reason the activity of the digestive enzymes was dependent upon duration of the larval exposure to the effect of potassium saline and what is the significance of different responses of the two digestive enzymes to the effect of that factor? Janković-Hladni *et al.* (1980) have shown that in fed as well as in unfed larvae of *M. funereus* kept under unfavourable conditions, protease and amylase show cyclical changes of different rhythm. It was assumed that cyclical changes in the digestive enzyme activity reflect the cyclical changes in the activity of the peptidergic neurons type A in the brain and the suboesophageal ganglion. It has been stated that in several insect species the peptidergic neurons type A show a circadian rhythm in their activity (Cymborowski and Dutkowski, 1969, 1970; Fujishita and Ishizaki, 1981, 1982). Thus, our results seem to suggest that the observed differences in the activity of the digestive enzymes, dependent on the exposure time of the larvae to saline, are the consequence of the different physiological state of the peptidergic A neurons in the brain and the suboesophageal ganglion during circadian and/or feeding rhythm. In addition, it was found that during prolonged exposure of the insects to the effect of solutions with high potassium concentration, the release of neurohormones was decreased (Nordmann and Morris, 1976; Maddrell and Nordmann, 1979).

Cerebral-complex extract

The results obtained under the effect of the cerebral-complex extract support the idea that in *M. funereus* larvae the parameters studied are under the control of neurohormones. Owing to their selective response to the effect of the extract, it might be assumed that they are regulated by several specific neurohormones or by different doses of the same neurohormone. Recent work has revealed that in addition to protease, a number of other digestive enzymes were regulated by one or more neurohormones (Muraleedharan and Prabhu, 1979, 1981; Raabe, 1982).

Our previous results have shown that the cerebral-complex extract, prepared in NaCl saline and injected into *M. funereus* larvae stimulates SPA and SAA (Ivanović *et al.*, (1978). In this context it should be recalled that the extract prepared in KCl saline stimulated or inhibited SPA and SAA, the obtained effect being dependent on the duration of the larval treatment with the extract. The response of SPA to the effect of the extract at various time intervals following injection gives the opportunity to gain insight into the mode of action of the gut hormone(s), presumably synthesized in peptidergic neurons type A. The significant decrease of SPA 12 h after the extract injection can be attributed to the additive effect of the extract and the saline, which in turn induces an over-release of the gut hormone(s). The extract-effect on SAA was reversed when compared with SPA, i.e. SAA was stimulated 24 h after injection.

Under the influence of the extract a decline in the haemolymph trehalose concentration was observed. Although the same trend concerning the larval response at the level of haemolymph trehalose and midgut protease was observed, the physiological significance of the responses is different.

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CHANGES IN CONCENTRATIONS OF IONS AND UREA IN BOTH PLASMA AND MUSCLE TISSUE IN A DEHYDRATED HYLID ANURAN

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Abstract—1. The rate of evaporate water loss of juvenile *Hyla arborea* was higher than that of adults. Both replaced most water during immersion for two hours but juveniles at a higher rate during the 1st hour.

2. Plasma concentration increased during dehydration from 200 to 510 mOsm/kg and maximum hematocrit during dehydration was 65%.

3. The change in plasma sodium was higher than potassium and vice versa in muscle. Both increased linearly with water loss. Changes in Cl^- concentration in both plasma and muscle behaved more like sodium than potassium.

4. Urea concentration during dehydration changed by about the same level in both plasma and muscle between 10 and 40 mM.

INTRODUCTION

Several anuran species are known to inhabit xeric habitats. The mechanisms that enable their survival under conditions of high temperature and low humidity were studied in several species (Bentley, 1966; Warburg, 1972). Some anurans, *Scaphiopus couchi* or *Bufo viridis* (see Bentley, 1966; McClanahan, 1967, 1972; Degani *et al.*, 1981), spend most of the year in burrows emerging only after rain when temperatures are low and relative humidity is high. The ability of dehydrated amphibians to absorb water from soil or when in water was determined in several species (Heatwole and Lim, 1961; Packer, 1963; Dole, 1967; Spight, 1967; Warburg, 1972; Warburg and Degani, 1979). The changes in body fluid (mainly plasma and urine) were studied in dehydrated frogs or when burrowed (Shoemaker *et al.*, 1969; McClanahan, 1972; Degani *et al.*, 1981) as well as in urodeles (Delson and Whitford, 1973; Degani and Shkolnik, 1979; Degani, 1981b, 1982). There is little information on the osmotic changes of anuran tissues during dehydration.

Hyla arborea is abundant in northern and central Israel, and can be found during the entire year including the hot and dry summer. In summer while clinging to leaves in moving air the danger of dehydration increases (Warburg, 1971, 1972).

In the present study we compare ions (Na^+, K^+, Cl^-) and urea concentrations in both plasma and tissues of dehydrated frogs. We therefore studied rates of dehydration and limits of water loss as well as rehydration in both juvenile and adult frogs. An attempt was made to establish relationships between the water content of both plasma and tissue ions (Na^+, K^+, Cl^-) and urea concentrations.

MATERIALS AND METHODS

Adult *H. arborea* were collected from winter ponds in the Galilee Mts. (Sasa), during the breeding period in February–March. Juveniles (300 ± 50 mg body wt) were collected in July after they had completed metamorphosis. The experiments were conducted in desiccators over dry silica gel (0–5% r.h.) at 10°C. Upper limits of water loss were determined when the frogs lost their co-ordination (Degani, 1981b). Five adults and five juveniles were used in each experiment. The adults were weighed every 10 hr, and juveniles every hour, until the frogs reached their upper limit of water loss. The water uptake was measured in both adult and juvenile frogs that had previously lost about 30% body weight, and were then placed in tap water. All weighing was carried out on a Mettler H315 balance (± 0.1 mg accuracy). Twelve adult tree frogs were dehydrated to different percentages of body weight to establish the effect of dehydration on concentration of both plasma and muscle. Blood samples were taken from the dehydrated frog by puncturing the heart, using 1 ml syringe previously washed with lithium heparin as described previously (Degani, 1981b). The blood samples were immediately centrifuged and the plasma frozen for later analysis. Muscle tissue from the hind leg was also taken. Water content in muscle tissue was determined by desiccating part of the muscle tissue at 60°C until it reached constant weight. Sodium, potassium and chloride concentrations in plasma and muscle tissue were determined after homogenation of muscle tissue using methods described by Brühmann and Hanke (1980) and Degani (1981b). Urea was determined by Foster and Hochholzer's (1972) method. Urea in muscle was measured after homogenation with TCA (5%) and immediate centrifugation for 15 min at 300 G. The statistical difference was analysed in each case by standard deviation, *t*-test, *d*-test and the correlation between the variability was analysed by regression and *r*.

RESULTS

The rate of evaporative water loss of juvenile *H. arborea* was significantly higher than that of adults (Table 1). Juveniles reached their limit of water loss after four days (at 10°C and 0–5% r.h.) while adults reached this point after about two weeks. The maximum limit of water loss was 45% for adults and about 35% for juveniles.

In both adults and juveniles most of the water uptake took place after 2 hr. There were no significant

Table 1. Evaporative water loss (at 10°C, 5% r.h.) and water uptake in juvenile ($n = 5$) and adult ($n = 10$) *Hyla arborea*

Time (hr)	Dehydration (in $mg/g^{-1} hr^{-1}$)	Time (hr)	Rehydration (in $mg/g^{-1} hr^{-1}$)
Juveniles			
1	160 ± 20	0.5	350 ± 80
2	110 ± 10	1	280 ± 80
3	90 ± 20	1.5	260 ± 70
		2	320 ± 50
Adults			
10	12.9 ± 2.3	1	5585.5 ± 171.0
20	9.0 ± 2.2	2	295.3 ± 101.1
30	6.9 ± 2.8		
44	6.4 ± 2.2		

differences between the two groups after that period. Juvenile frogs absorbed water quicker than adults, but the difference between them was significant after 1 hr (Table 1).

Plasma concentration increased following dehydration as is shown by the linear correlation between body wt loss and increased plasma concentration (Fig. 1). Maximum plasma concentration was 510 mOsm/kg in dehydrated frogs (that lost 45% of their body wt due to muscle dehydration). During the increase in plasma concentration the hematocrit rose up to 65% (Fig. 2).

Plasma Na^+ and K^+ concentrations increased linearly to the loss of water from the muscle. The change in plasma Na^+ was higher than that in K^+ (Fig. 3). The slope for Na^+ as indicated by the regression (calculated formula $y = 8.7 \times 109.1$) was greater than the slope of the formula calculated for K^+ ($y = 1.2 \times 2.7$). The opposite situation was found in muscle fluid during dehydration (Fig. 4). The slope calculated for K^+ ($y = 6.3 \times 124.4$) was greater than the slope calculated for Na^+ ($y = 3.4 \times +28.8$).

Plasma and muscle Cl^- concentrations were similar to those of Na^+, both followed a similar pattern (Figs 5 and 6). The slopes of plasma (5.3) and muscle (1.6) Cl^- concentration was much lower than the slopes of Na^+ in plasma (8.7) and in muscle (6.3) (see Figs 3 and 4).

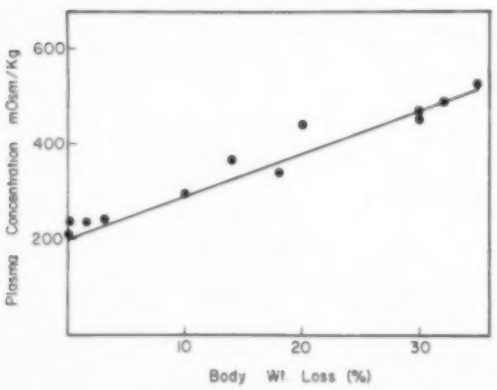

Fig. 1. Change of osmolarity in blood plasma of *Hyla arborea* during dehydration. $C = 8.8B + 199.2$ (C = plasma concentration in mOsm/kg. $B = \%$ body weight loss. $r = 0.92$; $P < 0.001$).

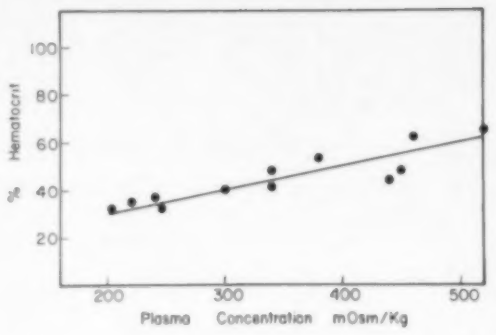

Fig. 2. Change of hematocrit as a function of increasing plasma concentration. $H = 0.09C + 12.7$ ($H = \%$ hematocrit. C = plasma concentration $r = 0.92$; $P < 0.001$).

Low urea concentration was found in both plasma and muscle (Figs 5 and 6). During dehydration urea concentration in muscle changed between 10 and 40 mM and the slope was 2.3 (Figs 5 and 6).

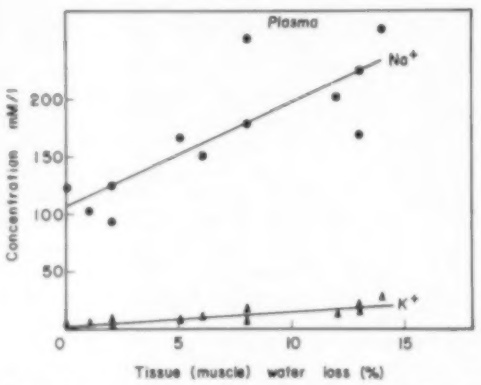

Fig. 3. Change of plasma Na^+ and K^+ concentrations as a function of tissue (muscle) water loss. Na^+, $C = 8.7W + 109.1$ (C = concentration in mM. $W = \%$ water loss in tissue). $r = 0.79$; $P < 0.01$. K^+, $C = 1.2W + 2.7$; $r = 0.87$; $P < 0.001$.

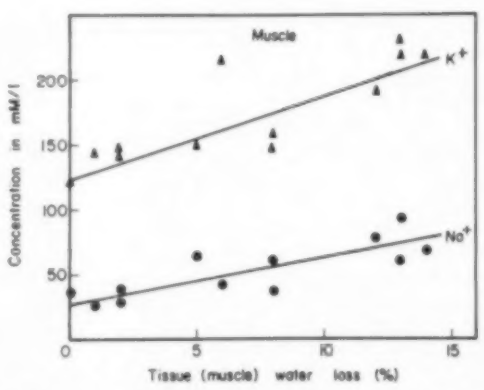

Fig. 4. Change of muscle Na^+ and K^+ concentrations as a function of tissue water loss muscle). Lettering as in Fig. 3. K^+, $C = 6.3W + 124.4$; $r = 0.85$; $P < 0.01$; Na^+, $C = 3.4W + 28.8$; $r = 0.83$; $P < 0.01$.

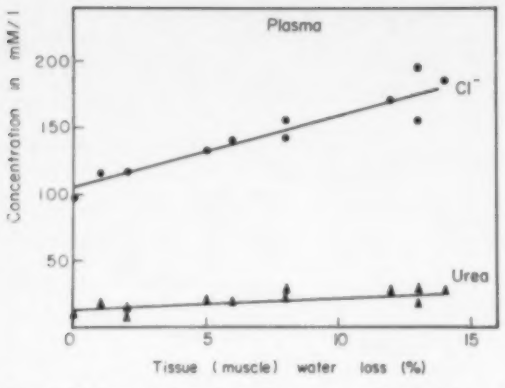

Fig. 5. Change in plasma Cl^- and urea concentration as a function of tissue water loss (muscle). Lettering as in Fig. 3. Cl^-, $C = 5.3W + 105.0$. $r = 0.93$; $P < 0.001$. Urea, $C = 1.1W + 12.4$. $r = 0.84$; $P < 0.01$.

DISCUSSION

In habitats that are comparatively xeric during 8 months of the year this small tree frog is found in danger of dessication (Warburg 1971; Warburg and Degani 1979; Degani and Mendelssohn 1979). It is known that water evaporates from amphibians at a rate similar to that from a free water surface.

This rate of water loss depends on both temperature and relative humidity (Warburg and Degani, 1979). The rate of water loss of adult *H. arborea* was higher than that of adult *B. viridis*, *Pelobates syriacus* and *Rana ridibunda* (Warburg, 1971). The ability to withstand dessication may vary considerably according to species and the degree of adaptation to terrestrial conditions. Both Thorson and Svihla (1943) and Schmid (1965) found that generally aquatic anurans are less tolerant to dehydration than species from drier habitats. Lethal water loss for the frog *Hyla moorei* was 31.4% body water, whereas for *Hyla caerulea* it was 45% body water (Main and Bentley, 1964). Hillman (1980) found that dehydration tolerance of six anurans species was as follows: 33.8% for *Xenopus laevis*, 37.0% for *Rana muscosa*, 37.3% for *Rana pipiens*, 41.4% for *Bufo boreas*, 42.9% for *Bufo cognatus*, and 44.9% for *Scaphiopus couchi*. In our study adult *H. arborea* survived 45% water loss, which is similar to that of anurans from more xeric habitats.

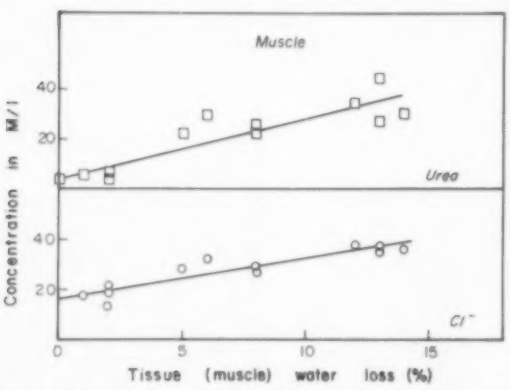

Fig. 6. Change of muscle Cl^- and urea concentration as a function of tissue water loss (muscle). Lettering as in Fig. 3. Cl^-, $C = 1.6W + 16.6$. $r = 0.93$; $P < 0.0001$. Urea, $C = 2.3W + 4.9$. $r = 0.88$; $P < 0.001$.

After dehydration amphibians are capable of absorbing water from wet surfaces (Heatwole and Lim, 1961; Packer, 1963; Warburg, 1967; Fair 1970). Our present study confirms earlier observations (Warburg, 1971) that the rate of water uptake was high in *H. arborea*.

Increased plasma concentration of *H. arborea* was found to be closely related to the loss of body water. A similar result was found in a toad *Bufo marinus* (Shoemaker, 1964) and Salamander *Salamandra salamandra* (Degani, 1981b). A positive correlation between hematocrit and blood volume was demonstrated in the sloth *Bradypus tridactilus* (Bozzini *et al.*, 1978). Similarly, a high hematocrit level was found in blood plasma of *S. couchi* (69%) following dehydration. In *H. arborea* hematocrit rose to similar levels (65%) following dehydration. Thus during dehydration plasma volume dropped whereas plasma concentration (which is due mainly to Na^+, K^+, Cl^- and urea) increased in a similar fashion as the hematocrit. The percentage of water in muscle decreased during dehydration while the concentration of Na^+, K^+, Cl^- and urea increased. The habit of *H. arborea* to stay on plants is due to its capability of tolerating a drop in plasma volume and increase in concentration of hematocrit, Na^+, Cl^- and urea.

On the other hand Na^+ concentration increased during dehydration to a higher level in plasma than in muscle, whereas K^+ increased more in muscle than in plasma. These results are in agreement with Alvarado's (1972) who studied the effect of dehydration on electrolytes in plasma and muscle of *Ambystoma tigrinum*.

Rapid dehydration of *H. arborea* caused mainly elevation of Na^+ and Cl^- in plasma and K^+ and Cl^- in muscle with only a small elevation in urea concentration. Mack and Hanke (1977) studied the elevation of Na^+, Cl^-, K^+ and urea during dessication in six anuran species. They found that the variation in urea concentration during dehydration was at its maximum 30 mM (in *B. viridis*) when plasma concentration rose and electrolytes increased.

In aestivating animals this mechanism is different after a long period of dehydration in soil. In *B. viridis*, urea concentration in the plasma rose to 68% and the osmolality to 1400 mOsm/kg when dehydrated for long periods (Degani *et al.*, 1981). This is similar to results found for *S. couchii* (73% increase in osmolality see McClanahan, 1972). *A. tigrinum* (40% see Delson and Whitford, 1973) and *S. salamandra* (35% in Degani, 1981a). In *B. viridis* kept in 80% seawater osmolality rose to 800 mOsm/kg when urea accounted for 36% of the plasma osmolality (Katz, 1973).

In conclusion *H. arborea* is capable of surviving in a xeric habitat by tolerating high rates of water loss and being capable of rapid replenishment of water. This becomes possible due to their ability to tolerate a decrease in volume of plasma and an increase in hematocrit. They are also able to tolerate a rise in plasma and muscle osmolality with increased elevation of Na^+, K^+ and Cl^-.

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TEMPERATURE AND METABOLISM OF PLANKTON—I. RESPIRATION OF ANTARCTIC ZOOPLANKTON AT DIFFERENT TEMPERATURES WITH A COMPARISON OF ANTARCTIC AND NORDIC KRILL

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(*Received* 20 *April* 1983)

Abstract—1. Direct response of 6 common Antarctic zooplankters (the euphausiid *Euphausia superba*, the copepods *Calanoides acutus* and *Euchaeta antarctica*, the amphipods *Parathemisto gaudichaudii* and *Hyperiella antarctica*, and the polychaete *Tomopteris carpenteri*) to temperature changes was measured and their temperature tolerance tested.

2. Respiration rates followed the Arrhenius equation in the range examined (generally −1 to +10°C).

3. Temperature coefficients varied between 8.06 and 23.84, while Q_{10} values from 1.64 to 5.24 were found.

4. When krill data were compared with experiments on North Sea *Meganyctiphanes norvegica*, no significant difference was found for Arrhenius coefficients.

5. No mortality occurred within 30 hr at temperatures from −1 to 10°C.

6. The results are discussed with regard to the concept of metabolic cold adaptation.

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INTRODUCTION

The interest in metabolic adaptation of polar ektotherms to low temperatures has a long history which has been summarized by Dunbar (1968) and Holeton (1974). The results of a comparative study of Arctic and tropical fish by Scholander *et al.* (1953) and of the work of Wohlschlag (1960, 1964) on Antarctic fish have been used to establish the concept of metabolic cold adaptation and this had been reviewed by de Vries (1977). It comprises elevated metabolic rates, obligate stenothermy and the loss of genetic acclimatization potential. The fact that Antarctic species cannot live at elevated temperatures would imply that their physiology and biochemistry differ markedly from those of temperate forms. However, Holeton (1974) criticized the elevated metabolic rates reported by Wohlschlag and suggested they were a result of experimental technique. In addition, subsequent experimental work has demonstrated, that metabolic rates in many polar marine invertebrates are low (see reviews Clarke, 1980, 1983) thus supporting Holeton's contention. Most of the data considered so far relate to fish and benthic organisms. This study deals with the effect of different temperatures on survival and respiration of six Antarctic zooplankters. It concentrates on short term reactions to immediate temperature changes. This is part of an ongoing project where acclimation to temperature and the role of enzymes in adaptation of polar zooplankton are being studied for comparison with plankton of lower latitudes. Thus the Nordic krill, *Meganyctiphanes norvegica*, was used here for comparison with the Antarctic krill, *Euphausia superba*.

MATERIAL AND METHODS

Species examined

The following six Antarctic zooplankters were examined: the euphausiid *Euphausia superba*, the copepods *Calanoides acutus* and *Euchaeta antarctica*, the amphipods *Parathemisto gaudichaudii* and *Hyperiella antarctica* and the polychaete *Tomopteris carpenteri*. *Hyperiella antarctica* has so far only seldom been found (R. Weigmann-Haass, personal communication). All the other species have a circumpolar distribution and are mainly restricted to the regions south of the Antarctic convergence. *Calanoides acutus* has also been reported from sub-Antarctic waters although specimens examined by Vervoort (1965) were thought to be moribund or dead when caught. *Parathemisto gaudichaudii* is the only bipolar species. In the Antarctic it occurs within the westwind drift and is present in both the Antarctic and sub-Antarctic zones. Its southern limit lies in the vicinity of the boundary between east and westwind drift. In the northern hemisphere there are records from the Arctic Ocean, the North Atlantic and the North-west Atlantic Ocean, but it is not clear whether it is the same species (see Kane, 1966). In addition to the Antarctic species, *Meganyctiphanes norvegica* was examined, an euphasiid confined to the North Atlantic and Mediterranean Sea (Mauchline and Fisher, 1969).

Collection of material

Material was collected during the Joint Biological Expedition on RRS *John Biscoe* in February 1982. Most live animals were caught with a ring trawl (1 m^2 opening, 2000 μ mesh size). A closed 5 l. jar served as codend to avoid damage to the material. The net was veered from the foredeck into the water surface for 1–5 min. *Euchaeta antarctica* were sorted out from the 1 m net of an RMT 1 + 8 m. *Calanoides acutus* was caught with a Nansen vertical net (200 μ). *Meganyctiphanes norvegica* was collected in the Gullmarfjord on the west coast of Sweden (max. depth 115 m) with a midwater trawl (1 m^2 opening, 500 μ). The Antarctic catch were immediately diluted with surface water while catches of *Meganyctiphanes* were diluted with deep water.

Experiments

The experimental methods should be easy to standardize for comparison with animals of different latitudes, and easy to conduct on board ship. As preliminary laboratory treatment

Table 1. Antarctic zooplankton: sampling time and locations with water temperature, incubation time and volume for respiration measurements, and dry weight of animals

Species	Date	Time (GMT)	Station	Depth (m)	Water temp. (°C)	Time of incubation (hr)	Incubation volume (ml)	Dry weight (mg/an)
1 *Euphausia superba*	23.2.82	03.00	63°27′S 56°43′W	0	−0.2	6	1/290	29–46
2 *Parathemisto gaudichaudii*	17.2.82	03.00	66°07′S 68°11′W	0	1.4	6	3/290	13–15
3 *Hyperiella antarctica*	14.2.82	03.00	63°45′S 65°42′W	0	1.1	6	3/110	3.5–4.5
4 *Tomopteris carpenteri*	14.2.82	03.00	63°45′S 65°42′W	0	1.1	6	1/290	71–95
5 *Euchaeta antarctica*	17.2.82	22.50	64°56′S 64°14′W	140–60	0.61–(−0.04)	6	3/110	4.4–5.0
6 *Calanoides acutus*	25.2.82	21.00	63°58′S 49°58′W	200–0	0.19–(−1.25)	12	7/60	0.58
7 *Meganyctiphanes norvegica*	23.9.81	10.00	58°20′N 11°30′E	100	6.5	6	1/290	97–105

may influence metabolism in an unpredictable way (Ikeda, 1977) especially under starvation, intact animals were immediately after sorting transferred into experimental flasks containing membrane filtered seawater (0.45 μ) of different temperatures. *Calanoides acutus* were used before temperature experiments in feeding experiments at 1°C for 22 hr (Schnack, in preparation). The flasks were incubated in the dark in thermostated water baths (± 0.5°C). After the experiment a subsample was siphoned off for oxygen determination by Winkler titration (Strickland and Parsons, 1972). Experimental animals were then kept in the remaining water for 24 hr and their survival tested at the experimental temperatures. Then they were rinsed with distilled water, stored at −20°C for transport home, dried to constant weight at 70°C, and weights measured.

Details of collection, environmental temperatures and experimental procedures are given in Table 1. Sampling locations are shown in Fig. 1.

RESULTS

Temperature tolerance

Although not acclimated all animals survived in the temperature tolerance experiments lasting at least for 30 hr at the respective temperature and were thus apparently far from their upper lethal temperature. All species reacted with raised swimming activity during the first seconds when brought into the higher temperatures except for *Calanoides actus*. This copepod did not show any reaction. In grazing experiments conducted prior to the temperature tolerance experiments they did not feed (Schnack, in preparation). They also had large oilsacs. These observations suggest that the copepodite stage V studied here was already in the inactive overwintering condition.

Respiration

When the reverse temperature values, expressed in degrees of the Kelvin scale, are plotted against the logarithms of the respiration rates, the dependence of respiration upon temperature can be satisfactorily represented by a straight line with the Arrhenius equation

$$V = V_0 \, e^{-\mu/RT}$$

where V is the respiratory rate, V_0 is a coefficient having the same measure as V, μ is the "temperature characteristic" i.e. the coefficient expressing the accelerative influence of temperature, R is the gas constant (1.986 kcal mole^{-1}), T is the absolute temperature (K).

Another measure of the influence of temperature upon biochemical and physiological reactions is the Q_{10} of van t'Hoff

$$Q_{10} = \left(\frac{R_2}{R_1}\right)^{10/(t_2 - t_1)}$$

with R_1, R_2 as respiration rates at the temperatures t_1, t_2.

In Table 2 the Q_{10} calculated for two temperature steps is listed together with the coefficients for the Arrhenius equation.

According to this function Q_{10} decreases with increasing temperature.

The temperature characteristic μ varies almost by a factor of three. There appear to be three groups with

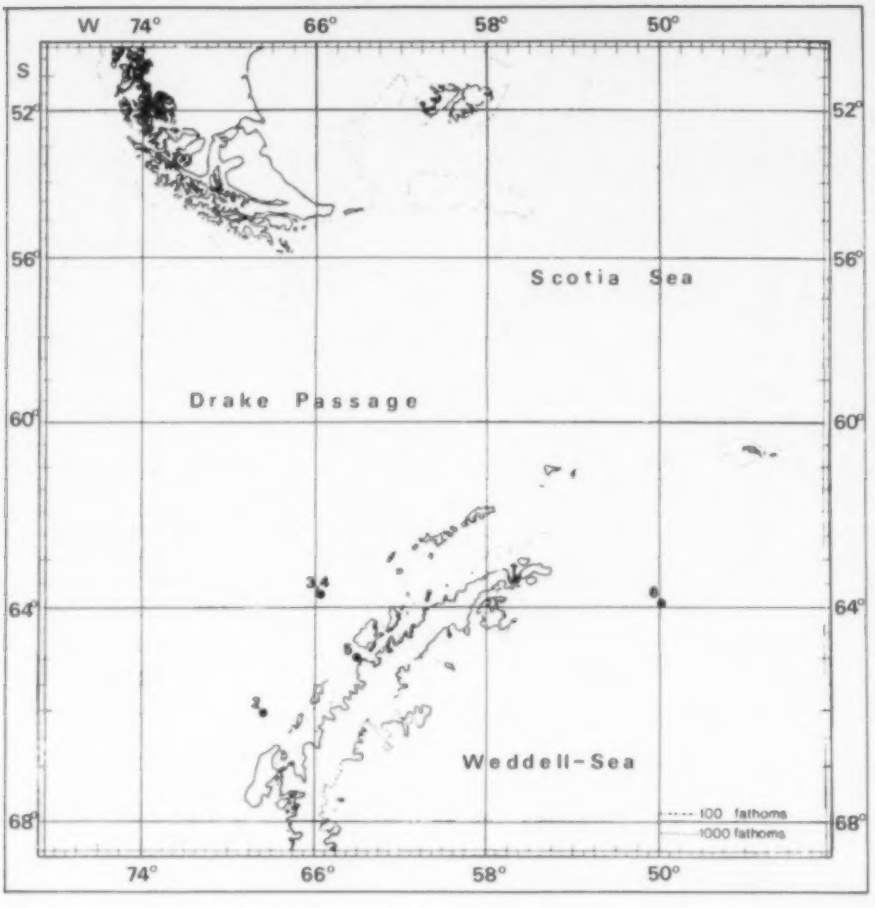

Fig. 1. Sampling locations. 1, *Euphausia superba*; 2, *Parathemisto gaudichaudii*; 3, *Hyperiella antarctica*; 4, *Tomopteris carpenteri*; 5, *Euchaeta antarctica*; 6, *Calanoides acutus*.

similar μ values:

I. *Euphausia superba*, *Meganyctiphanes norvegica*, *Hyperiella antarctica*;
II. *Parathemisto gaudichaudii*, *Tomopteris carpenteri*; and
III. *Euchaeta antarctica*, *Calanoides acutus*.

It is noteworthy that for taxonomic groups like copepods and euphausiids the μ values are similar, but not so for the amphipods.

When the μ values of *Euphausia superba* and *Meganyctiphanes norvegica* were compared according to the comparison of regression coefficients (Sachs, 1978, p. 341), no significant difference was found ($P = 0.01\%$).

DISCUSSION

Significance of methods

In studies concerning the response of animals to temperature changes 3 general time periods are distinguished (Prosser, 1973):

direct response of rate functions to changes in temperature persisting for hours;
compensatory acclimations during days or weeks of exposure; and
selection over generations of genetic variants adapted to a certain temperature regime.

This study deals only with direct responses to temperature changes in poikilotherms. The initial

Table 2. Antarctic zooplankton: coefficients of Arrhenius equations, correlation coefficients and Q_{10}

Species	a	b	r	μ	Q_{10} 0–5	Q_{10} 5–10
Euphausia superba	27.817	−7.727	0.97	15.35	2.80	2.62
Meganyctiphanes norvegica	26.459	−7.360	0.98	14.62	2.62	2.53
Hyperiella antarctica	26.571	−7.455	0.99	14.81	2.64	2.53
Parathemisto gaudichaudii	18.597	−5.157	0.96	10.25	1.96	1.88
Tomopteris carpenteri	14.030	−4.057	0.98	8.06	1.68	1.64
Euchaeta antarctica	45.0	−12.782	0.99	23.84	5.57	5.24
Calanoides acutus	36.859	−10.692	0.98	21.24	4.08	3.80

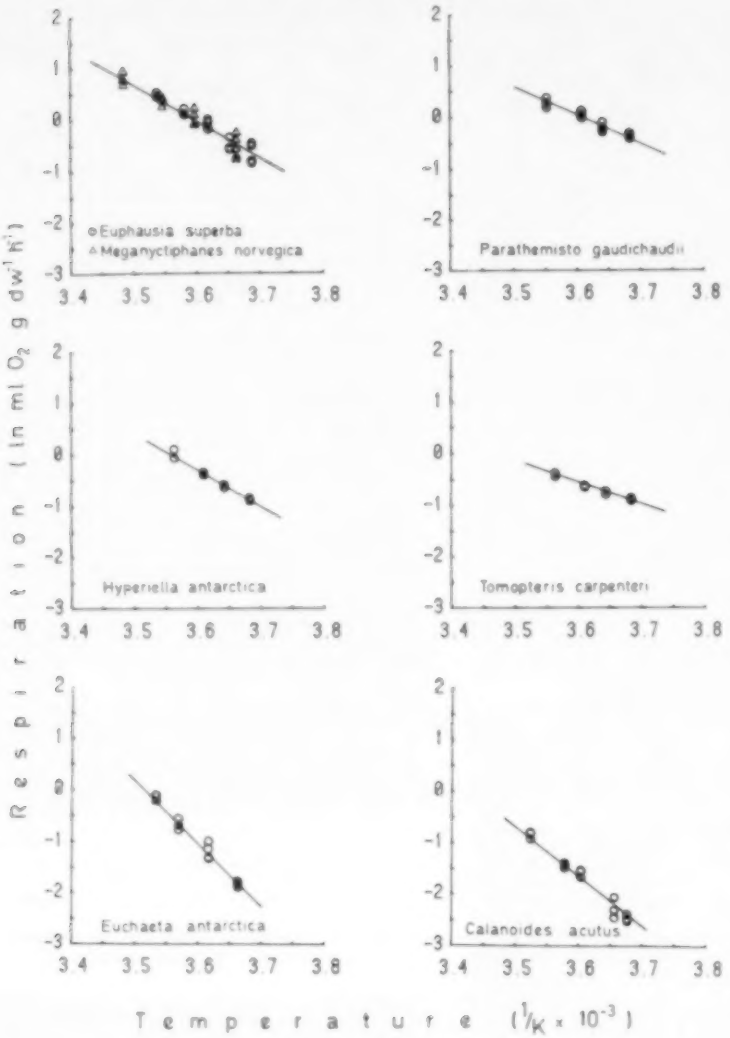

Fig. 2. Arrhenius plots for respiration rates of Antarctic zooplankton.

period after the temperature change is extremely unstable, metabolic rates undergo many changes (Ivleva, 1973). This may be accompanied by an overshoot followed by a period of stabilization which takes place over a period of minutes or hours (see Precht *et al.*, 1973).

Other difficulties concern the determination of the level of metabolism of zooplankton (see also Clarke, 1983) particularly the discrepancy between "closed" and "throughflow" respiration measurements and how these relate to true *in situ* respiration. However, since this study was aimed at comparing respiration rates between species rather than determining absolute values closed bottle respirometers are therefore suitable.

Respiration

Metabolic comparison with previous studies at field temperature. The oxygen uptake data of previous workers on Antarctic zooplankton and nordic krill are listed in Table 3. There is some variation in the data for the respective species. Some authors emphasized on different weight specific respiration rates for size groups with higher rates for smaller individuals. Inconsistent results between different workers may relate to differences in the method of collection at sea, pretreatment in the laboratory and experimental container size. The values obtained here fit well into the range of previous data except for the two copepod species. Their respiration is lower in comparison to the literature data. With regard to *Calanoides acutus* this may relate to the observation, that these animals were apparently in overwintering condition, where oxygen consumption is drastically reduced as was found for *Calanus hyperboreus* (Conover, 1962) and *Calanus finmarchicus* (Hirche, 1983).

Respiration and temperature. All respiration–temperature relations measured here follow the Arrhenius equation. This has been taken as a criterium for full acclimation by Ivleva (1973). With regard to the experiments of Shcherbakov (1935) and Marshall *et al.* (1935), that yielded Arrhenius equations without special control of the completion of the acclimation process, the same author relates this to the possible

Table 3. Respiration rates (OC, ml O_2/gdw per hr) of Antarctic zooplankton

Species	OC	Temp (°C)	Reference
Euphausiids			
	0.69	−1	McWhinnie and Marciniak (1964)
	0.50	5	Chekunova and Rynkova (1974)
	0.46	1.5	Rakusa-Suszczewski and Opalinski (1978)
	0.90	1	Kils (1979)
	0.188–0.388	−1.5	Segawa *et al.* (1979)
	0.382	−0.5	Ikeda and Hing Fay (1981)
Euphausia superba	0.349–0.486	−1	Ikeda and Mitchell (1982)
	0.67	1	Voss (1982)
	0.193–1.228	?	Segawa *et al.* (1982)
	0.63	1	Hirche (1983)
	0.62	0.8	Hirche (this study)
	1.27	13	Mayzaud (1973)
	1.77	5	Sameoto (1976)
Meganyctiphanes norvegica	0.27*	5	Teal and Carey (1976)
	0.49–0.91	5	Båmsted (1979)
	1.18	5	Voss (1982)
	1.05	5	Hirche (this study)
Copepods			
Calanoides acutus ♀	0.398	−0.5	Ikeda and Hing Fay (1981)
Calanoides acutus CV	0.086	−1	Hirche (this study)
Euchaeta antarctica	0.742*	2	Biggs (1982)
Euchaeta antarctica ♀	0.160	0	Hirche (this study)
Calanus propinquus ♀	0.406	−0.5	Ikeda and Hing Fay (1981)
Calanus propinquus	1.13	−1	Ikeda and Mitchell (1982)
Metridia gerlachei	1.0	−1	Ikeda and Mitchell (1982)
Rhincalanus gigas	0.47	−1.7	Rakusa-Suszczewski *et al.* (1976)
Amphipods			
Parathemisto gaudichaudii	0.450	−0.5	Ikeda and Hing Fay (1981)
Parathemisto gaudichaudii 3.6 mg	0.739	−1	Ikeda and Mitchell (1982)
Parathemisto gaudichaudii 13.12 mg	0.424	−1	Ikeda and Mitchell (1982)
Parathemisto gaudichaudii	0.680	−1.5	Hirche (this study)
Orchomene plebs†	0.94	−1.8	Rakusa-Suszczewski *et al.* (1976)
Paramoera walkeri†	0.283	−1.8	Rakusa-Suszczewski and Klekowski (1973)
Orchomenella sp.	0.343*	2	Biggs (1982)
Vibilia antarctica	0.50	−1.1	Ikeda and Mitchell (1982)
Hyperia gaudichaudii	0.213	−0.8	Ikeda and Mitchell (1982)
Hyperiella antarctica	0.420	−1.5	Hirche (this study)
Polychaets			
Tomopteris carpenteri 9.73 mg	0.457	−1	Ikeda and Mitchell (1982)
Tomopteris carpenteri 138.0 mg	0.310	−0.8	Ikeda and Mitchell (1982)
Tomopteris antarcticus	0.410	−1.5	Hirche (this study)

* Assuming dry wt = 26% wet wt.
† Benthic.

adaptation of plankton to a great range of temperatures as in natural conditions it shows considerable vertical migration. However, searching in the literature for further Arrhenius equations for the metabolism–temperature relationship in plankton, only the data of Paranjape (1967) fit into the equation. In Table 4 the values of μ and Q_{10} of this study are compiled together with literature data. When the μ values are compared, differences are highest among copepods. The Antarctic species have a temperature coefficient almost twice that of the boreal species *Calanus finmarchicus*. μ values of the three euphausiid species are not significantly different ($P = 0.01\%$). Much more data are available for Q_{10}. Most of the values lie within the normal biological range of 2–3. Q_{10} values as high as those found for the two copepod species in this study were only reported for the amphipod *Paramoera walkeri* (Klekowski *et al.*, 1973). It is not clear whether these high values indicate an erroneous behaviour or some kind of stress as is generally suggested for Q_{10} higher than 5. According to Ivleva (1973) high Q_{10} values are the result of too short acclimation periods to the thermic conditions of the experiment. An extremely low Q_{10} was found by McWhinnie and Marciniak (1964) for Antarctic krill in acute measurements. This was not found again by subsequent workers on this species, however, it is reported for several benthic organisms. The amphipod *Orchomene plebs* showed a decrease in respiration rate between −1.8 and +1°C and only a slight increase at 4–7°C (Rakusa-Suszczewski, 1982). Belman and Giese (1974) investigated the effect of temperature on respiration of *Sterechinus neumeyeri* (Echinoidea) and *Odonaster validus* (Asteroidea) acclimated for 3 days at temperatures ranging from −1.8 to 3°C. No significant differences were found in oxygen consumption rates of both species at the examined range of temperatures. Wohlschlag (1960, 1963) found a wide range of respiration rates at the lowest temperature and no significant increase with raised temperatures.

Temperature tolerance

A contrasting trend of eurythermy in Arctic species and stenothermy in Antarctic species has been sug-

Table 4. Antarctic zooplankton: temperature coefficient (μ) and Q_{10} data from this study and the literature

Species	μ	Q_{10}	Temp (°C)	Acclimation time	Reference
Euphausiids					
	15.4	2.8	0–5	no	This study
		2.36	−1–5	4 hr	Voss (1982)
Euphausia superba		2.26	−1–1.5	24–48 hr	Rakusa-Suszczewski and Opalinski (1970)
		1.1–1.3	0–5		McWhinnie and
		2.1	5–15	no	Marciniak (1964)
Meganyctiphanes norvegica	14.6	2.51	5–10	no	This study
Meganyctiphanes norvegica		1.81	5–10	4 hr	Voss (1982)
Meganyctiphanes norvegica		1.88	5–10	no	Teal and Carey (1967)
Euphausia pacifica	13.8	2.21	5–10	24 hr	Paranjape (1967)
Copepods					
Calanoides acutus CV	21.2	4.08	0–5	no	This study
Euchaeta antarctica ♀	23.8	5.57	0–5	no	This study
Calanus hyperboreus		1.76	2–8	no	Conover (1961)
C. finmarchicus ♂	12.9	2.33	0–5	18 hr	Marshall *et al.* (1935)
C. finmarchicus ♀	8.8	1.80	0–5	18 hr	
C. finmarchicus CV	12.9	2.35	0–5	18 hr	
Amphipods					
Parathemisto gaudichaudii	10.3	1.96	0–5	no	This study
Hyperiella antarctica	14.8	2.64	0–5	no	This study
*Paramoera walkeri**		15.9	−1–0	18–24 hr	Klekowski *et al.* (1973)
		4.6	0–2		
Polychaets					
Tomopteris carpenteri	8.1	1.68	0–5	no	This study

* Benthic.

gested by de Vries (1977) and George (1977). This has been ascribed to the old age of the Antarctic ecosystem, where "stenothermy became imprinted in an ineffaceable way in the genotpye of endemic Antarctic species" (George 1977). In Table 5 the upper incipient lethal temperatures of Antarctic animals are listed. Compared with data for Arctic species presented by de Vries (1977, Table II), the Antarctic species are in fact stenotherm. McWhinnie and Marciniak (1964) report the only value for Antarctic zooplankton, *Euphausia superba*. In their experiments animals placed into 4°C and above became quiescent, lost their glassy transparency and died within 24 hr. This is at variance to the present results. These differences cannot be ascribed to different sensitivity of life stages to higher temperature as was found by Rakusa-Suszczewski (1982) for Antarctic amphipods, since the animals used by McWhinnie and Marciniak were only slightly larger (30–60 mg/an) than those used in this study (29–46 mg/an).

The good condition of the animals after the experiments, despite direct exposure to high temperatures, and the linearity of the Arrhenius plot up to the highest temperature make assume, that they were far from their upper incipient lethal temperature. It thus appears that Antarctic zooplankton is not as stenotherm as other Antarctic marine organisms.

Conclusions

The present observations on Antarctic zooplankton do not support the concept of metabolic cold adaptation. Instead, respiration rates followed the

Table 5. Upper incipient lethal temperature (UILT) of Antarctic animals

Species	UILT (°C)	Reference
Euphausiids		
Euphausia superba	4	McWhinnie and Marciniak (1964)
Amphipods		
Orchomenella chilensis	12	Armitage (1962)
Orchomene plebs	10	Rakusa-Suszczewski and Klekowski (1973)
Orchomene plebs	8	Rakusa-Suszczewski (1982)
Paramoera walkeri	7–8	Rakusa-Suszczewski (1981)
Isopods		
Glyptonotus antarcticus	4	George (1977)
Echinoderms		
Sterechinus neumeyeri	5	Belman and Giese (1974)
Odonaster validus	5	Belman and Giese (1974)
Fish		
Trematomus bernachii	6	Somero and de Vries (1967)
Trematomus hansoni	6	Somero and de Vries (1967)
Rigophila dearborni	6–7	de Vries (1977)
Trematomus borchgrevinki acclimated to 4°C	6–7	de Vries (1977)
	7–8	de Vries (1977)

Arrhenius equation over the temperature range studied. There were no Q_{10} values found as low as reported for *Euphausia superba*, fish and benthic organisms by previous workers.

When the temperature coefficient and Q_{10} were compared between the Antarctic krill, *Euphausia superba*, which lives preferentially at temperatures between 0 and 3°C (Weigmann-Haass and Haass 1980), the Nordic krill, *Meganyctiphanes norvegica* (2–21°C: Casanova, 1974) and *Euphausia pacifica* (5–20°C: Brinton, 1962; Paranjape 1967), no significant difference was found.

Antarctic zooplankton apparently has a higher temperature tolerance than Antarctic benthos and fish. The question arises therefore for an exceptional position of zooplankton. This could relate to the higher temperature variations to which plankton is exposed. However, the present experiments do not allow conclusions concerning adaptation capacity. The problem of an elevated metabolism of Antarctic zooplankton in comparison to plankton of other latitudes warrants further research.

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PHOTOPLETHYSMOGRAPHY—A NON-INVASIVE TECHNIQUE FOR MONITORING HEART BEAT AND VENTILATION RATE IN DECAPOD CRUSTACEANS

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Abstract—1. A new, non-invasive technique is described for monitoring heart rate and scaphognathite activity in decapod crustaceans.

2. The photoplethysmograph is commercially available, inexpensive and operates equally well in air or water, in the laboratory and *in situ*.

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INTRODUCTION

A number of techniques are available for monitoring heart rate and ventilation rate in decapod crustaceans. The simplest approach is to observe cardiac activity and respiratory movements directly or with the aid of a binocular microscope. However, it is often desirable to make long term recordings from unrestrained animals and for this more elaborate techniques are required. Ventilation rate and heart rate are usually monitored by recording pressure fluctuations (Blatchford, 1971) or by impedance techniques (Ansell, 1973; Uglow, 1973; Depledge, 1978). A third possibility, electrocardiography is available for monitoring cardiac activity (Florey and Kriebel, 1974). Unfortunately, to obtain recordings either a hypodermic needle or electrodes need to be implanted in or attached to the animal. This often takes several minutes and the resulting trauma may invalidate physiological measurements for at least 24 hours.

A new technique is described which allows heart rate and ventilation rate of certain decapod crustaceans to be monitored non-invasively both in air and water. The procedure involves the use of cheap, commercially available, photosensitive detectors and potentially has widespread applications.

TECHNIQUE

The Pulse Plethysmograph (Bioscience Ltd., Harbour Estate, Sheerness, Kent, ME12 1RZ, UK) was developed to enable heart rate of infants to be monitored during anaesthesia. The device is shown in Fig. 1. It consists of two small bulbs emitting low intensity light and an extremely sensitive photosensor. For use with aquatic animals it must be dismantled and the electrical components embedded in transparent resin. The dimensions of the package can be reduced to 1.5 × 1.5 × 0.5 cm and its weight to 10 g in air although it is weightless in water.

To measure heart rate of decapod crustaceans (large prawns, shrimps, crabs and lobsters) the photosensor is attached to the cardiac region of the carapace with a water-resistant, instant adhesive (Eastman 910-Kodak). This can be accomplished in seconds with minimal disturbance to the animals. Low intensity light from the bulbs passes through the dorsal carapace into the pericardium (Fig. 2). During the heart's cycle of action varying amounts of light are reflected by the ventricle. Some of this light falls on the photosensor which converts the energy into a proportional voltage change to operate an oscillograph (George Washington MD2, Bioscience Ltd.). When recordings have been completed the device is readily detached leaving the animal undamaged.

The same principle can be used to measure ventilation rate. The gills of decapods are ventilated by the rhythmic beating of gill bailers (scaphognathites). If the detector is positioned on the anterioventral surface of the carapace, under the scaphognathite, ventilatory movements can be monitored (Fig. 3).

VERIFICATION OF THE TECHNIQUE

Although the intensity of light emitted by the bulbs in the plethysmograph is extremely low, photoplethysmography might influence heart rate directly, for example, as a light stimulus to cardiac tissue; or indirectly as a result of a local heating effect. These possibilities were investigated.

A crab (*Carcinus maenas*—carapace width = 6 cm) was prepared for invasive impedance cardiography. The details of this technique have been given elsewhere (Ansell, 1973; Uglow, 1973; Depledge, 1982). Briefly,

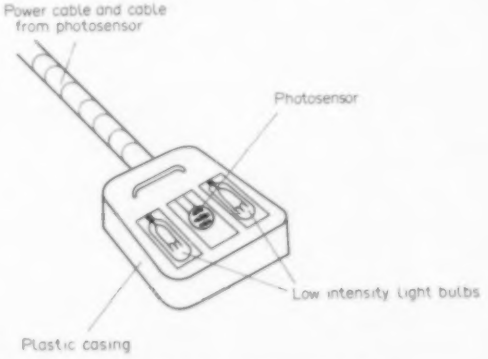

Fig. 1. The photoplethysmograph.

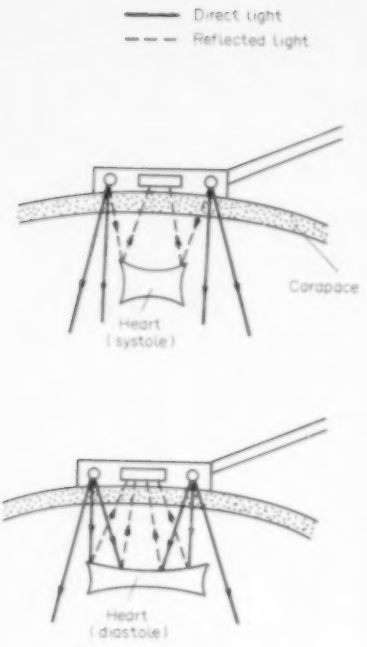

Fig. 2. Principle of operation of the photoplethysmograph.

electrodes are implanted on either side of the heart. They are connected to an impedance cardiograph (Scientific Instruments Centre) and heart rate is displayed on an oscillograph (George Washington MD2, Bioscience Ltd.). The photoplethysmograph was then fitted between the electrodes allowing cardiac activity to be recorded by the two techniques simultaneously (Fig. 4). Individual heart beats correlated precisely as did periods of cardiac arrest. No noticeable change in heart rate nor trace height was observed when the light source of the plethysmograph was switched on or off. Furthermore, when the light source was left on for long periods (30–60 min) there was no significant change in heart rate nor trend of increasing rate. It was therefore concluded that photoplethysmography allows individual heart beats to be monitored whilst having no effect on cardiac activity. The above procedure was repeated while measuring ventilatory movements. Typical traces are shown in Fig. 5. Again the technique apparently had no effect on scaphognathite rate.

DISCUSSION

Photoplethysmography is a cheap and extremely simple way of recording cardiac and ventilatory activity in decapod crustaceans. It is applicable in air and in water as well as during passage from one medium to the other. Successful recordings have been

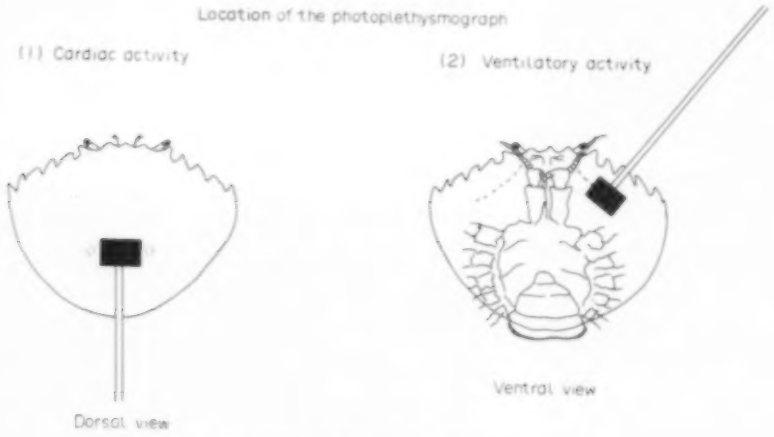

Fig. 3. Location of the photoplethysmograph.

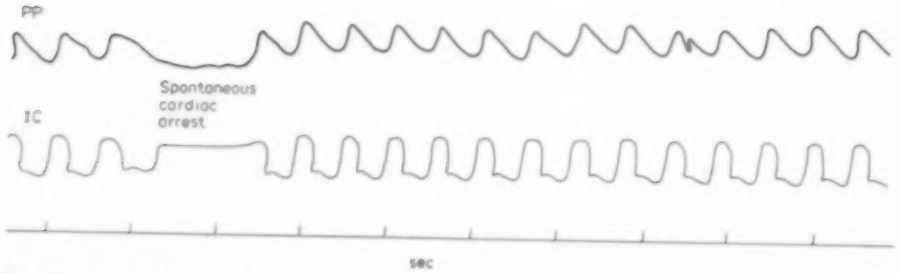

Fig. 4. Correlation of monitoring techniques—cardiac activity. PP, photoplethysmograph; IC, impedance cardiograph.

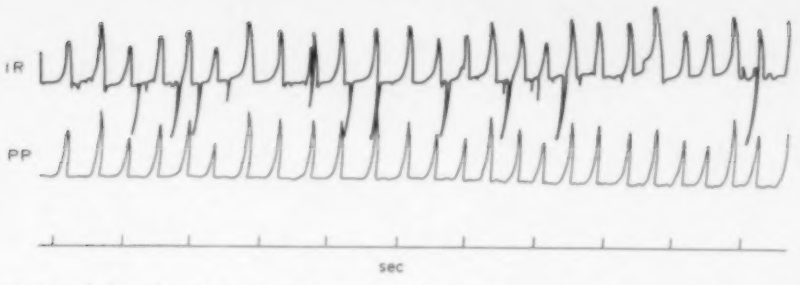

Fig. 5. Correlation of monitoring techniques—scaphognathite activity. PP, photoplethysmograph; IR, impedance recording.

obtained from a variety of crabs (*Carcinus*, *Macropipus*, *Sesarma*, *Uca*, *Ocypode*), from large prawns and shrimps (heart rate only) and from the Norwegian lobster, *Nephthrops norvegicus*. However, there is no reason why other animals would not be suitable subjects, for example, thin-shelled bivalve molluscs, pipefish etc. Larger subjects are easier to attach the device to than small ones but recordings can easily be obtained from crabs with a carapace width of as little as 2 cm.

Other significant advantages of the technique are that it is non-invasive, leaving the subjects completely intact and permitting long term recordings to be made with ease. Also, because the photoplethysmograph can be attached in seconds with a high degree of certainty that recordings will be obtained, handling of the animal is minimal. This renders the technique particularly suitable for use on the seashore and a portable version is now being designed.

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EFFECT OF TEMPERATURE ON THE VENTILATORY RESPONSES OF THE EURYTHERMIC LIZARD *LEIOLOPISMA NIGRIPLANTARE*

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(*Received* 3 *May* 1983)

Abstract—1. The ventilatory responses of the eurythermic skink *Leiolopisma nigriplantare* (= *zelandica*) were studied over the body temperature range 0–40°C. Ventilatory rates of resting lizards were acutely measured and electropneumograms (EPG) were analyzed.

2. Mean ventilatory rates increased from 7.4 to 78 breaths/min (5–40°C) corresponding to a Q_{10} of 1.9. Ventilatory activity below 5°C is generally sporadic, although ventilation was observed as low as −1.3°C. Although tachypnoea occurs under conditions of heat stress, these lizards do not pant.

3. The electropneumic cycle consists of a double burst of high frequency electrical activity associated with active expiration and active inspiration, separated by an electrically quiescent period. The effect of temperature on these events were determined over the range 0–20°C.

INTRODUCTION

Ventilatory responses of lizards and other reptiles have been studied extensively, but not often over as wide a range of body temperatures as they would normally encounter in the field or as they can tolerate. In addition, ventilatory responses of lizards capable of activity over a broad temperature range (i.e. eurythermic) and at low temperatures have not been adequately studied.

In a previous study (Morris, 1971), it was observed that the electrical activity of the respiratory muscles (electropneumogram) of a skink could be readily recorded from the body surface. This activity had been previously described for the iguanid *Crotaphytus collaris* (Dawson and Templeton, 1963) and is undoubtedly recordable in other lizard species. There has apparently been no attempt, however, to investigate this activity with respect to temperature.

The objective of this study, therefore, was to characterize the ventilatory response of a small eurythermic lizard over the range 0–40°C in terms of ventilatory rate and electropneumic events.

MATERIALS AND METHODS

Experimental subjects

The common or garden skink *Leiolopisma nigriplantare* (= *zelandica*†) *maccani* is probably the most numerous and ubiquitous of the New Zealand reptiles. This skink is widely distributed altitudinally (from sea level to at least 1200 m) and latitudinally (to 47°S, hence, among the southernmost lizards in the world) in New Zealand.

On the South Island, *L. nigriplantare* lives in a variety of habitats: on the eastern shore (where the skinks in this study were obtained), in city gardens and inland on the plains and mountains. During the winter in some of these habitats, substrate and air temperatures fall below the freezing point of saurian tissues. Whereas in the summer, substrate temperatures on the sand dunes of Kaitorete spit, may reach 50°C which is well above this skink's maximum lethal limit. By burrowing under vegetation and debris, *L. nigriplantare* and other sympatric lizards are not generally exposed to these extremes.

On the basis of field and laboratory studies, *L. nigriplantare* is clearly eurythermic (Morris, 1974). Body temperatures (BT) measured in the field when most skinks were active ranged from 13.6 to 32.9°C: $\bar{X} = 25.2$°C ($N = 35$). Laboratory observations indicated that *L. nigriplantare* is capable of voluntary locomotion at BT as low as −1°C, and that it can tolerate acute exposure to BT as low as −6°C (at any time of the year) and as high as 41°C (in the summer only).

The skinks used in this study were captured by hand at Kaitorete Spit which is about 40 km south of Christchurch, South Island. At this spit, the lizards inhabit young sand dunes which are sparsely covered with the sand-stabilizing sedge, *Desmoschoenus spiralis* (pingao). These skinks are readily collected on any fine day throughout the year as they move rapidly among the pingao when their cover is disturbed.

After capture, the skinks were kept in an outdoor, sand-filled terrarium (9 m²) on the roof of the Zoology Building of the University of Canterbury where they were exposed to the prevailing weather and photoperiod of the austral summer. The nutritive status of the skinks used in this study—judging by their alertness, celerity and amount of fat storage—was excellent.

Experimental design

Subadults (about 1–2 years) and adult male and post-partum females were used, ranging 40–65 mm in snout–vent length and 1.3–5.7 g in weight.

All temperatures in this study were measured with thermistors calibrated against a Braun "Warburg" thermometer in a Grant thermostatic bath and are accurate to within 0.1°C.

Ventilatory responses were observed at six "temperature stations" in the range 0–40°C to facilitate statistical analyses of the data. Ventilatory rates for resting skinks were acutely measured after at least 30 min of equilibration (except at 40°C). Since *L. nigriplantare* tolerates 40°C for only 15–20 min, the rates were obtained when this temperature was reached.

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† The specific name was changed by Hardy (1977).

Electropneumography

From a previous study (Morris, 1971), an orthogonal YY lead, consisting of two platinum electrodes placed in the longitudinal axis, was found to be more satisfactory for recording the electrical events associated with ventilatory and cardiac activity in unanesthetized skinks than other lead positions (McDonald, 1976). The active or jugular electrode was implanted subcutaneously about half-way between the forelimb and the eye opening, and ventral to the thoracic musculature. The indifferent or cloacal electrode was inserted with a thermistor into the cloaca.

The subjects were taped onto balsa wood stretchers that were designed to keep the leads of the electrodes and thermistors in place. The electrodes were connected push-pull to Tektronix AX preamplifiers where the signals were amplified about 1000×. An 80 Hz cutoff was employed to reduce the presence of the lower frequency cardiac signals. The output from the preamplifiers were fed into a sound amplifying system and/or a Hewlett–Packard recording system. The ventilatory rate could be obtained by counting the sounds directly or by recording EPG events at a low paper speed.

The subjects were placed into a temperature cabinet where their body temperatures could be maintained to within 0.1°C of the desired level. A different set of subjects were used for the temperature stations 0–35°C and 40°C. For convenience, the former experiments were run 20–35°C, then 0–20°C. The subjects were monitored during at least the last 5 min of equilibration to assure that they were inactive prior to recording. The duration of EPG events were measured from high speed paper recordings using vernier calipers and a stereo microscope.

RESULTS

Ventilatory responses to temperature

Ventilatory rates for resting skinks were acutely measured between 0 and 40°C. When the data are plotted semilogarithmically (Fig. 2), a line can be fitted by eye for the range of 5–40°C with a slope corresponding to a Q_{10} of 1.9. The Q_{10} values, however, vary considerably for the intervals within this temperature range: 2.9 (5–10°C), 1.7 (10–20°C), 2.0 (20–30°C), 1.3 (30–35°C) and 2.6 (35–40°C). In the range 5–40°C, ventilation of resting skinks occurs at fairly regular intervals. At 0°C, however, ventilation generally consists of a few breaths (at 15–30 sec intervals) followed by apnoea lasting for several minutes and sometimes as long as 10 min. For this reason, no mean ventilatory rate was determined at 0°C.

Typical of other scincids (Dawson, 1960; Hudson and Bertram, 1964), the ventilatory rate changes only slightly between 30 and 35°C, but at higher temperatures there is a substantial increase in the rate. But under such conditions of heat stress, skinks neither pant as occurs in some iguanids (Dawson and Templeton, 1963), nor gape without panting as occurs in anguids (Dawson and Templeton, 1966) and agamids (Bartholomew and Tucker, 1963).

Using an orthogonal YY lead, it is possible to record the electrical activity of the intercostal muscles of *L. nigriplantare* which is associated with ventilation. Such records are referred to as electropneumograms (EPG). As illustrated in Fig. 1, EPG typically consist of double bursts of high frequency electrical activity that correspond to active expiration and active inspiration separated by an electrically quiescent period (EQP). After a period of apnoea, ventilatory activity is initiated by active expiration, followed immediately by passive inspiration (due to the elastic recoil of the ribs) during which no electrical activity of the intercostal muscles is detected and coincident with the EQP. After the discrete period of electrical quiescence, active inspiration occurs followed immediately by a partial expiratory movement (also passive and due to the elastic recoil of the ribs). An EQP associated with the final event of the ventilatory sequence is continuous with the period of apnoea between breaths. To facilitate analyses of EPG, general muscular (EMG) and extraneous noise levels had to be minimal, and the lower frequency signals associated with cardiac activity (ECG) were filtered out. At temperatures above 20°C, however, ECG signals increased considerably in amplitude, rate and in high frequency components, obscuring the boundaries of the EPG cycle. Consequently, EPG have been analyzed only between 0 and 20°C. For clarity, only the mean values of these events are plotted semilogarithmically in Fig. 3.

The durations of active expiration and active inspiration are not significantly different ($P > 0.1$) and

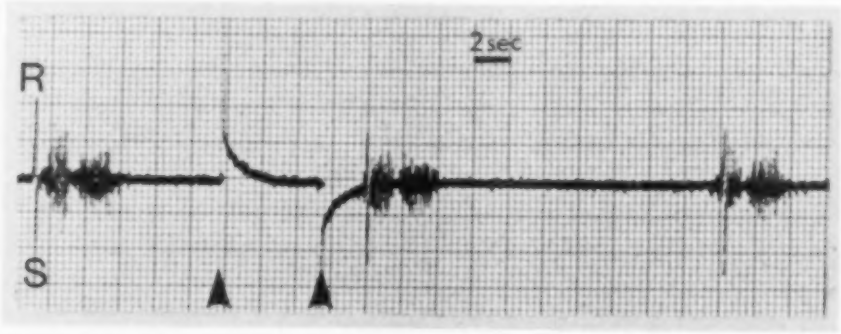

Fig. 1. Electropneumogram (EPG) at −1°C illustrating the double bursts of electrical activity associated with active expiration and active inspiration, separated by an electrically quiescent period (EQP). The subject is only slightly supercooled. Ventilatory and cardiac (indicated by the RS wave) rates are nearly equal at about 3.4 per min. The low frequency cutoff was at 80 Hz. A square wave inserted during apnea (between arrows) and the RS waves show electrical differentiation.

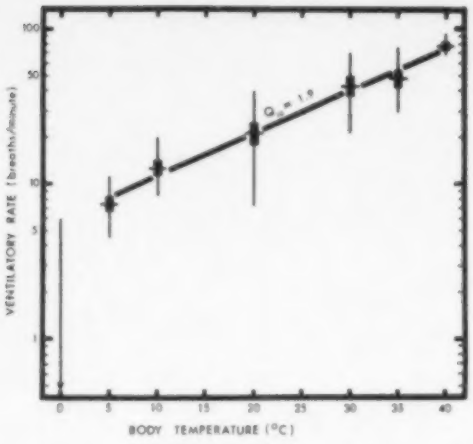

Fig. 2. Relation of ventilatory rate to body temperature. Data represent acutely-measured values obtained after 30 min equilibration, except at 40°C. At least 20 subjects were used at each temperature station, except at 40°C (N = 10). The range is represented by the length of a vertical line, the mean by a horizontal bar and 2 SE on each side of the mean (equivalent to about 95% confidence limits) by a shaded rectangle. The range at 0°C extends down to 0.1 breaths/min.

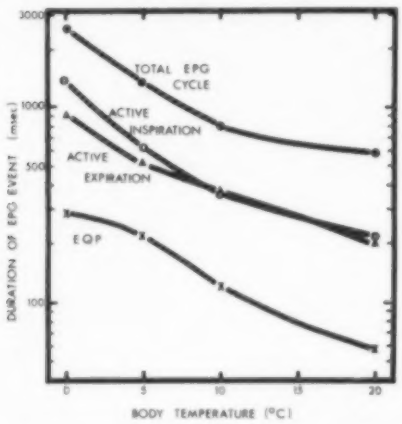

Fig. 3. Relation of durations of electropneumographic (EPG) events to body temperature. The electrically quiescent period (EQP) occurs between the periods of active expiration and active inspiration (see text). For clarity, only the means are given.

constitute about equal portions of the EPG cycle. The intervening EQP of the cycle occupies about 12% of the total duration of the EPG cycle. The Q_{10} values for the reciprocals of the EPG events are presented in Table 1.

Table 1. Temperature coefficients (Q_{10} values) for EPG events (reciprocals of durations)

Events	Temperature interval (°C) 0–5	5–10	10–20
Total EPG cycle	3.5	2.7	1.7
Active expiration	3.1	1.9	1.9
Electrically quiescent period (EQP)	1.6	3.3	2.2
Active inspiration	4.7	3.0	1.7

DISCUSSION

Ventilatory response to temperature

Although the effect of temperature on the ventilation of lizards and other reptiles has been extensively studied, generally only the ventilatory rate is analyzed. The small size of *L. nigriplantare* precluded measurement of tidal and minute volumes which is a more precise measure of ventilation. Ventilatory rate is temperature-dependent as occurs for many other physiological parameters, but the response is often more complex with considerable individual variability (Templeton, 1970). Eurythermic scincids such as *Eumeces obsoletus* (Dawson, 1960) and *Leiolopisma* species (Hudson and Bertram, 1966; Morris, 1971; present study), exhibit low thermal dependence (i.e. their Q_{10} values approach unity) over a 5–7 degree interval. This contrasts with other lizard species for which there is little or no change in rate over a 10–15 degree interval (Dawson and Templeton, 1963, 1966; Burns, 1971). These intervals occur within a species' activity or preferred temperature range.

The ventilatory rate does not fall off abruptly until the body temperature drops below about 5°C for the eurythermic *Leiolopisma* species and the eurythermic anguid *Gerrhonotus multicarinatus* (Dawson and Templeton, 1966). This is in sharp contrast with the ventilatory response of more thermophilic lizards, the stenothermic heliophiles. Ventilation in these lizards is very slow and irregular at 10–15°C commensurate with their general lethargy at these body temperatures (Dawson and Templeton, 1963; Boyer, 1967). Under heat stress, however, eurythermic scincids like *L. nigriplantare* breathe very rapidly, with a ventilatory cycle resembling the eupnoea of endothermic vertebrates. With the diminution of the period of apnoea between passive expiration and active expiration, the ventilatory rate can be increased considerably without a substantial change in the duration of the individual EPG events that make up the cycle (see below). The tachypnoeic response exhibited by skinks under heat stress is not, however, the same as the panting response described for heliophilic iguanids and agamids.

The electropneumic response to a range of body temperatures has been previously reported only for *L. lineoocellatum* (Morris, 1971) although only the total duration of electrical activity was analyzed. The trends in this response are similar for the two *Leiolopisma* species, but the duration of EPG events are longer for the larger-sized skink, *L. lineoocellatum*. The electropneumic cycle illustrated for the stenothermic heliophile *Crotaphytus collaris* at 25°C (Templeton and Dawson, 1963) has a duration comparable to that obtained for the *Leiolopisma* species at 0°C. Since the iguanid *C. collaris* weighs 25–40 g, weight differences account for some of this disparity.

Limited data for *L. nigriplantare* and *L. lineoocellatum* above 20°C suggest that the EPG events have low thermal dependence (i.e. the Q_{10} values approach unity). Consequently, the higher ventilatory rates at high temperatures are primarily achieved by shortening the period of apnoea between breaths rather than by reducing the time required for active expiration and inspiration.

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MECHANICAL PROPERTIES OF MUSCLES FROM *XENOPUS BOREALIS* FOLLOWING MAINTENANCE IN ORGAN CULTURE

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Abstract—1. The mechanical properties of sartorii muscles from the toad *Xenopus borealis* were studied in organ cultures lasting up to 26 days.

2. Isometric twitch and tetanic tensions diminished in force reaching half their initial values after 15 days in culture. In contrast, twitch time to peak time to half relaxation, twitch–tetanus ratio and muscle weight–body weight ratio, were unchanged.

3. Maximum isotonic shortening velocity (V_{max}) declined, with a half time similar to that of the isometric force.

4. The fall in isometric tension is probably due to a breakdown of activation in some fibres. The change in V_{max} could be due to a loss of functional sarcomeres in series with the tendons.

INTRODUCTION

The effects of denervation of muscles *in situ* have been followed, but in addition to nerve loss the muscles are subject to other factors, for example the strains imposed by the activity (or denervation) of other muscles and the influence of other physiological systems via the intact blood supply. By following the changes in isolated muscles it should be possible to examine the effects of denervation in a controlled environment.

Harris and Miledi (1972) described a technique for culturing amphibian muscle. They found that frog muscles could be kept for up to 2 months without major changes in ultra structure. Glycogen granules were seen in abundance. Nuclei, mitochondria and sarcoplasmic reticulum had their usual organisation. Pinocytotic vesicles similar to those seen in fresh muscles were observed, presumably indicating the persistence of active exchange of material between muscle and extracellular fluid. These authors studied the electrophysiological characteristics of the muscles during the first 2 weeks of culture, and found that resting and action potentials were near normal during this period although neuromuscular transmission failed after 5–7 days.

In the present experiments the mechanical properties of muscles kept under culture conditions similar to those of Harris and Miledi (1972) were investigated in order to see if mechanical function is also preserved. There is a suggestion from work on mammals that mechanical function may not be as well preserved as electrophysiological function. Purves and Sackmann (1974) working on cultured rat diaphragm muscles found that the muscle twitch became weaker after 2–4 days, whereas the resting membrane potential was well maintained for 9 days.

A preliminary report of this work has appeared in the Proceedings of the Physiological Society (McDonagh, 1982).

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METHODS

Dissection and culture technique

Toads (*Xenopus borealis*) weighing 7–34 g were used because, unlike frogs, they fed themselves readily. Well fed animals are essential for long survival of muscles in culture (Harris and Miledi, 1972). The physiological properties of *Xenopus borealis* muscle are indistinguishable for those of the more commonly used (Lannergren, 1979) *Xenopus laevis* (M. J. N. McDonagh, unpublished observations). The details of the culture methods were very similar to those described by Harris and Miledi (1972), but briefly the sartorius muscle was dissected out under aseptic conditions inside a laminar flow operating bench. The animal was stunned, decapitated and pithed. It was then submerged for 5 sec in 70% alcohol in order to kill bacteria on the skin surface. The skin covering the legs was cut around the waist and pulled off. Fresh sterile scissors were then used for the subcutaneous dissection. The superficial fascia along the lateral edge of the muscle was cut through and a 50 mm piece of cotton was tied to the distal tendon of the muscle which was then freed from its insertion. The muscle was lifted clear of the leg using blunt dissection. Short lengths of cotton were tied to the medial and lateral sides of the connective tissue plate to which the proximal end of the muscle is attached. The plate was then cut free from the body.

After dissection the muscle was tied to a glass gibbet. The gibbet and muscle were then placed in a Pyrex glass chamber holding 90 ml of sterile culture medium (Fig. 1, see figure legend for further details). Once the muscle and gibbet had been inserted, the top of the chamber was plugged with sterile non-absorbent cotton wool and covered with a loosely fitting lid of aluminium foil. The chambers containing the muscles were kept at room temperature (between 18 and 21°C) in a clean cabinet.

The totally defined culture medium 199 (Morgan *et al.*, 1950) was adapted for use with frog tissues by altering the inorganic ionic concentrations to those usual for frog Ringer solution (Harris and Miledi, 1972). This left the organic constituents at half their normal concentrations. Harris (1968) found that this did not alter the survival time and physiological activities of the muscles. The final ionic concentrations of the culture media were NaCl, 94 mM; KCl, 2.7 mM; $CaCl_2$, 2.0 mM; $MgSO_4$, 3.0 mM; Na_2HPO_4, 0.5 mM;

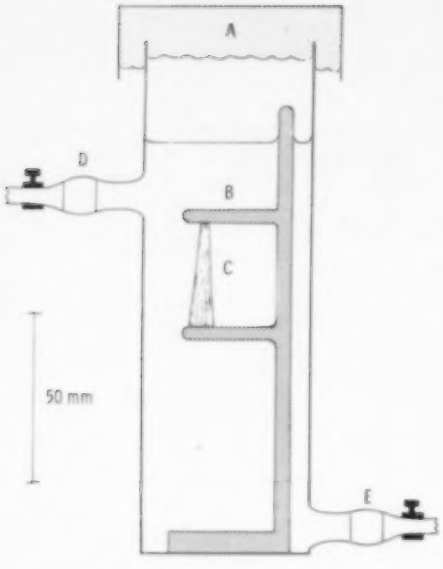


Fig. 1. Glass culture chamber A: non-absorbent cotton wool covered with aluminium foil lid. B: glass gibbet. C: Sartorius muscle. D and E: outlet and inlet pipes.

NaH_2PO_4, 3.6 mM; $NaHCO_3$, 2.0 mM; pH 7.6; osmotic pressure, 219 mosmols; ionic strength, 0.12. Other metabolites added to the medium were insulin (0.1 IU/ml), glucose (22 mM) and glutamine (2 mM). The antibiotic chloramphenicol (5 μg/ml) and the fungicide funigizone (2 μg/ml) were also added.

Mechanical recording

At the end of the culture period a muscle was removed from the chamber and gibbet, and its proximal end was attached to a Perspex holder. Holder and muscle were then immersed in a glass chamber (Blinks, 1965) full of oxygenated culture media at 20°C. The distal end of the muscle was attached to a force transducer (Statham, Type U.F.1 4 oz, resonant frequency 450 Hz). The output of the transducer was amplified and then displayed on an oscilloscope. Isotonic tetanic concentrations were also measured using a length transducer (T1 transducer, Washington Instruments) modified to use a reversed-biased diode as the sensor. This gave an improved signal/noise ratio, linearity and rate of response. The length transducer included a 65-mm-long dural lever of mass 348 mg and lever ratio 1:10. The muscle was attached to the long arm of the lever. Weights were attached to the short arm of the lever by means of a 670-mm-long, 2-mm-dia elastic thread. The long lever ratio and the compliant load attachment reduced the equivalent mass of the rotating system to 139 mg.

The muscles were stimulated with 1 msec pulses whilst immersed in culture media. In initial experiments the addition of D-tubocurarine (1.5×10^{-5} M) did not affect the tension produced by supramaximal stimulation. These pulses were provided by a stimulator capable of delivering up to 400 mA. Current flowed parallel to the long axis of the muscle between four 10-mm-dia hoops of platinum wire fixed at right-angles to this axis. Pulse frequency and pulse duration were controlled by a gated pulse generator and Digitimer. Twitches and tetani were evoked using twice maximal voltages with the muscle at a length at which twitch force was maximal. Tetanic stimulation consisted of trains of these pulses at 125 per sec for 400 msec. Twitches and tetani were given every 2 min during both the isometric and isotonic measurements. With this rest interval fatigue was not a problem.

RESULTS

The progress of denervation induced changes in the mechanical properties of *Xenopus* sartorius muscles was investigated by taking measurements from muscles after periods in culture ranging from 4 to 26 days. Figure 2 shows typical myograms of isometric twitches and tetani from a control muscle and from a muscle removed from culture after 26 days.

Table 1 summarises the values of the mechanical measurements taken from uncultured toad sartorius muscles. Also included for comparison are the similar results obtained by Lannergren (1979) using single fibres and by Ridge and Thomson (1980) using whole *Xenopus* muscle. The data indicate that the main fibre type present is Lannergren's Type 2 fibre.

The muscles were weighed after the mechanical measurements were taken (i.e. at the end of the culture period). The ratio muscle weight/body weight (mg/g) showed no change with time in culture. The value of 2.9 ± 1.0 (mean $\pm$ SD) for cultured muscles was the same as the value of 2.9 ± 0.8 (mean $\pm$ SD) obtained for control muscles. As is common with denervated amphibian twitch muscle, fibrillation was not observed in any of the cultured muscles.

Changes in twitch and tetanic tension are illustrated in Fig. 3, which shows that within 15 days twitch and

Table 1. *Xenopus* twitch muscle: normal values of mechanical properties

	T_p (msec)	$T_{1/2}$ (msec)	T_w (mN)	T_t (mN)	(T_w/T_t)	V_{max} (muscle length/sec)
This work						
Mean	32.8	31.5	124	268	0.44	7.95
SEM	1.7	2.1	18.8	24	0.04	1.17
N	15	15	15	14	14	4
Lannergren (1979)						
Type 2 fibre	34.3	34.8	*	*	0.61	5.2
Type 4 fibre	117	151	*	*	0.21	1.1
Ridge and Thomson (1980)	39.6	34.8	*	*	0.37	—

The table shows the mechanical properties of normal uncultured *Xenopus* twitch muscle. Values from the literature are included for comparison. Measurements in these experiments and in Lannergren's work were taken at 20°C. Ridge and Thomson's results were at 22°C. Ridge and Thomson's results are from whole extensor digitorum Longus IV muscles. Lannergren's results are from single fibres.

Abbreviations: T_p, twitch time to peak; $T_{1/2}$, time to half relaxation from peak twitch tension; T_w, twitch tension; T_t, tetanic tension; V_{max}, velocity of unloaded shortening.

*Data comparisons inappropriate.

—Data not available.

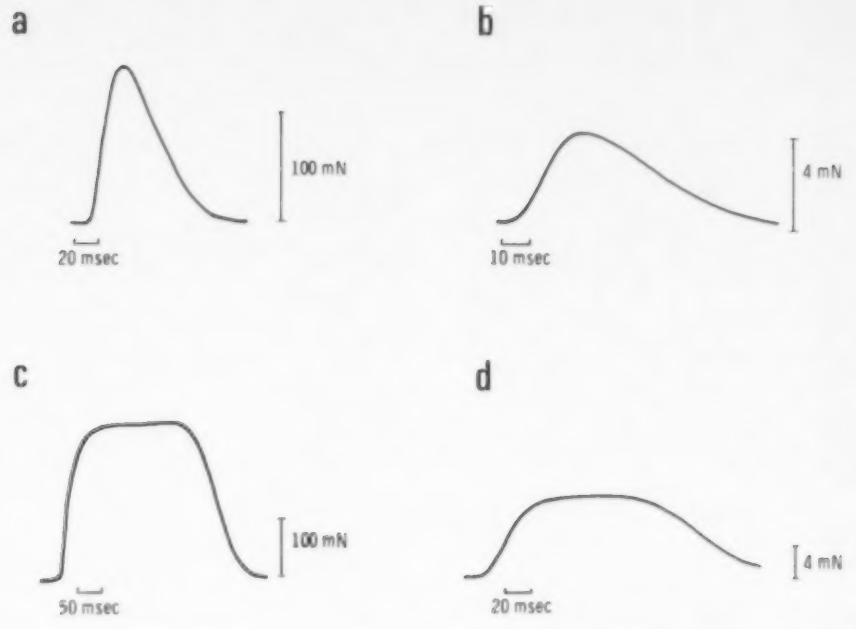


Fig. 2. Isometric myograms: A and C from a control muscle; B and D from a muscle culture for 26 days. A and B twitches. C and D tetani (stimulation at 125 Hz). Records have been retouched for clarity.

tetanic tension had dropped to 50% of its original value. Twitch–tetanus ratio and time to peak tension (Fig. 4) show no progressive change with time in culture (see figure legends). This was also true of time to half relaxation. Most values are within one standard deviation of the mean control value at time zero. This is in complete contrast to the tension data shown in Fig. 3.

Measurements of isotonic tetanic contractions were also made at a series of loads. Figure 5 shows isotonic myograms and below force–velocity curves taken after 0, 13 and 22 days in culture. The maximum velocity of contraction showed a progressive reduction with time in culture, as can be seen from Fig. 6 (open circles). Velocities at all other loads were similarly reduced. This contrasts with the lack of change in the time course of the isometric twitch and the twitch–tentanus ratio.

The tetanic force of isometric contractions was reduced to around 20 N towards the end of the culture period (Fig. 3). The tetanic force of such a muscle is indicated in brackets at 20 days in Fig. 6. However, there were muscles cultured for short periods which had similar low tensions, for example at 5 days (Fig. 6). Despite its low tension this muscle had a similar velocity to a muscle at 6 days with a tetanic force of 100 N. This indicates that the progressive reduction in the maximum velocity of contraction of the cultured muscle is not due to an interaction between the low tetanic tension of the cultured muscles and the inertia of the recording system. The contribution of inertia was checked in the following way. Whole fresh sartorius muscles were divided longitudinally into strips which had tetanic tensions similar to that of cultured muscles. The maximum velocity of contraction of these strips was measured. The measured maximum velocities of the weaker strips was reduced in a systematic way as a result of the inertia of the lever system. A plot of V_{max} of strip/V_{max} of whole muscle vs tetanic tension enabled an inertial correction factor to be calculated. This has been applied to the points (filled circles) in Fig. 6, and it can be seen that inertial effects do not explain the reduction of contraction velocity in culture.

DISCUSSION

According to Harris and Miledi (1972) muscles cultured under conditions similar to those described in these experiments appeared normal when examined under the electron microscope (see Introduction). It is, therefore, unlikely that the loss of muscle force described here is associated with gross structural changes in the muscles such as loss of myofibrils. Part of the loss of tension could be due to a reduction of the muscle action potential. Harris and Miledi (1972) found a reduction of the action potential from 128 mV to 95 mV in muscles from poorly fed animals. However, this did not occur in well fed animals as those used in my experiments. It is also unlikely that the loss of force could be due to failure of neuromuscular transmission in any remaining nerve endings because, as curarization showed, the pulse width used was sufficient for direct stimulation of the muscle fibres. The fact that the time to peak and time to half relaxation of the twitch were unchanged suggests that the time constants of calcium release and reuptake by the sarcoplasmic reticulum were unaltered. The most likely cause of the loss of force is progressive all or none failure of action potential propagation in the sarcolemma or in the t-tubules.

In the present experiments the time course of twitch contractions of amphibian muscle did not change following denervation and culture *in vitro*. This result contrasts with the findings in mammals. In the rat both

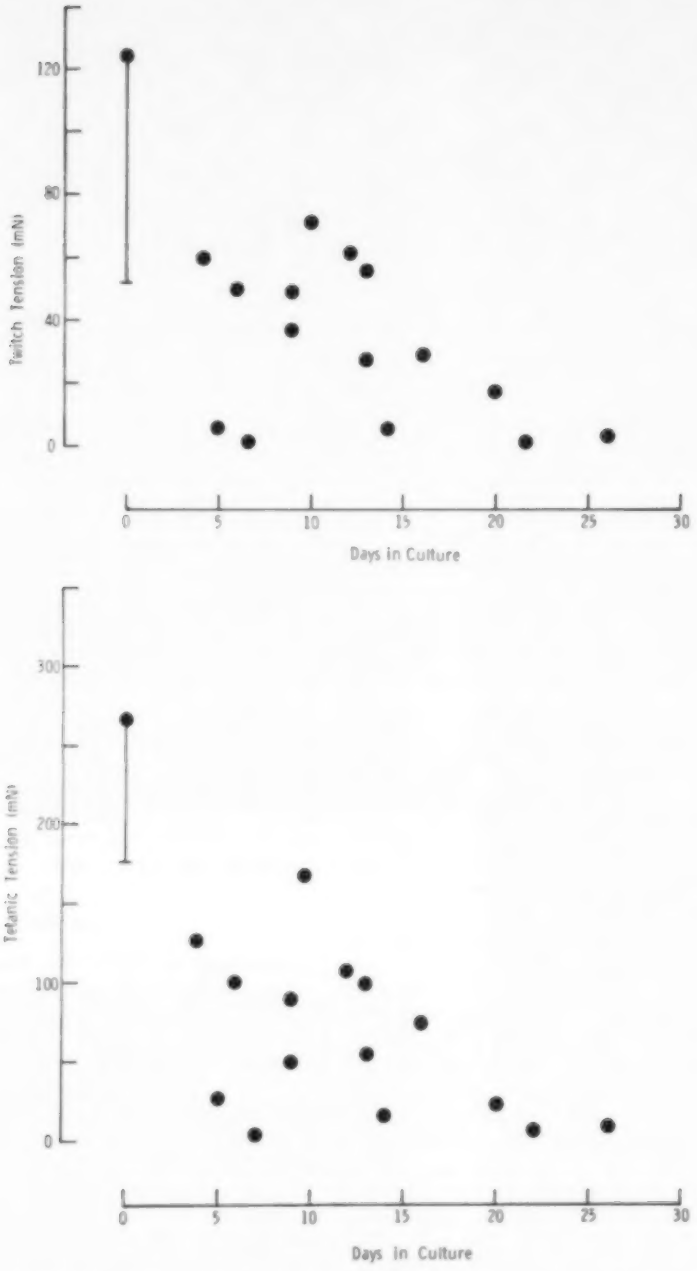
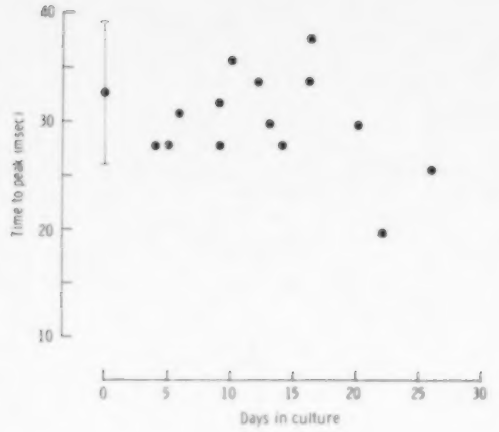


Fig. 3. Above: twitch tension changes in cultured muscle. The linear regression coefficient of the points (including 0 days). $r = -0.77$ ($P < 0.01$). The point at 0 days represents the mean of 15 control muscles. The bar indicates 1 SD. Each of the other points represents one muscle. Figures 4 and 5 are organised in a similar way.

Below: tetanic tension changes, stimulation at 125 Hz. $r = -0.71$ ($P < 0.01$).

E.D.L. and soleus muscle showed an increase in time to peak and time to half relaxation following denervation *in vivo* (Finol *et al.*, 1981). These changes were virtually complete after 4 days. Assuming a Q_{10} for the process of 2–2.5, equivalent changes at 20°C might be expected to be complete after 13–18 days in my experiments. No such changes were found. It has been suggested (Finol *et al.*, 1981) that the increase in time to peak found in denervated mammalian muscle is due to the reduced amplitude and prolongation of the muscle action potential which occurs at the same time. The fact that an equivalent change in muscle action potential does not occur in cultured amphibian muscle (Harris and Miledi, 1972) could well explain the lack of change in



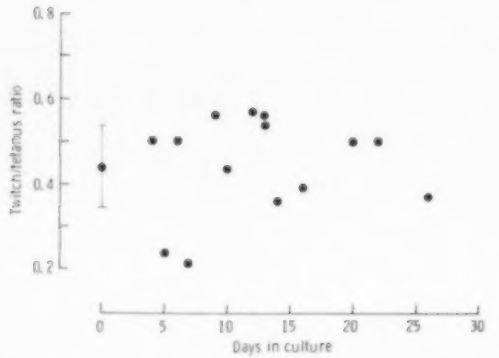
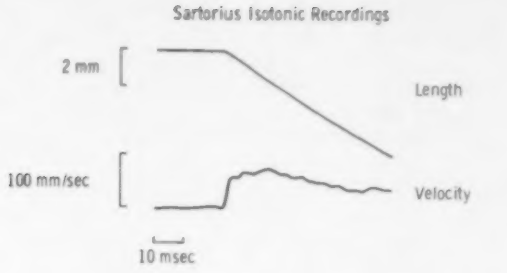


Fig. 4. Above: time to peak twitch tension of cultured muscles. $r = -0.15$ ($P > 0.1$).

Below: twitch–tetanus ratios of cultured muscles. $r = -0.03$ ($P > 0.1$). These variables show no progressive change during culture.

the time course of the twitch found in the present experiments. The time course of the twitch does change in cultured mammalian muscle (McDonagh, 1980).

If all or none failure of action potential propagation in the sarcolemma or t-tubules is occurring in the cultured muscle, this would fit well with the finding that there is a progressive reduction of maximum velocity of shortening but no change in the time course of isometric contractions. If the all-or-none failure affected individual half-sarcomeres the number of active sarcomeres in series would be reduced with a consequent reduction in muscle shortening velocity. Inactive half-sarcomeres would increase the compliance of the muscle, thus tending to increase time to peak of the twitch and to decrease twitch–tetanus ratio. This was not observed, so any such inactive half-sarcomeres must be stiffer than those of normal non-active muscle. This could occur if they were in rigor. An



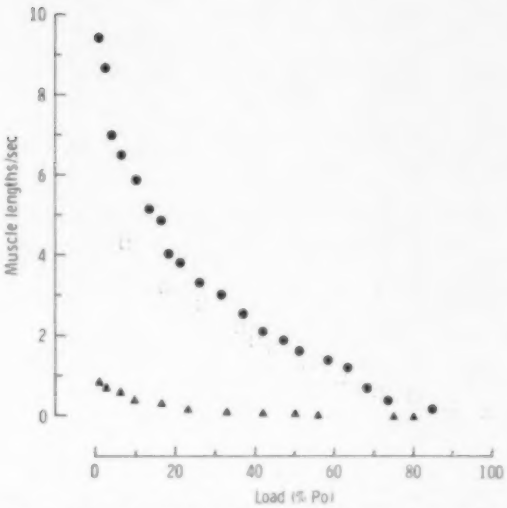


Fig. 5. Above: isotonic myograms from control sartorius muscle contracting against a load equal to 25% of isometric tetanic value.

Below: force velocity curves of cultured muscle. Results from three different muscles. Load expressed as percentage of isometric tetanic force.

●, control muscle (muscle length, 22 mm); □, after 13 days in culture (muscle length, 20.5 mm); ▲, after 22 days in culture (muscle length, 23 mm).

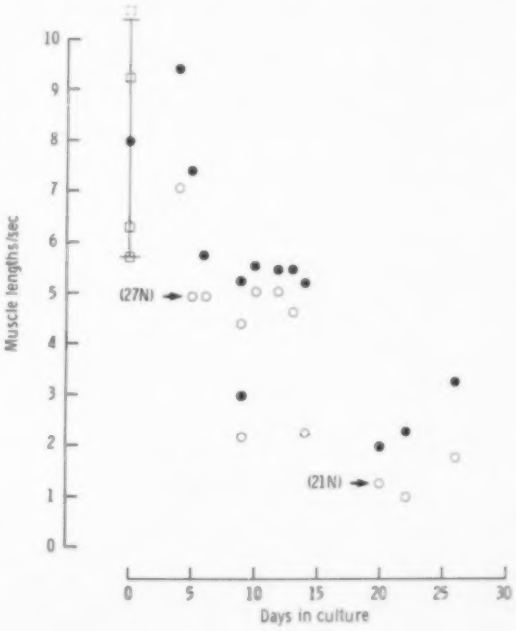


Fig. 6. Maximum isotonic contraction velocity of culture muscle.

□, control values (the mean of these is shown (○) ± 1 SD); ○, uncorrected points (each point represents one muscle); ●, points corrected for inertia ($r = -0.840$, $P < 0.01$). The figures in brackets are the tetanic forces of two muscles (see text).

alternative explanation is that there is a structural reduction in the number of sarcomeres in series in the cultured muscle. This might arise because muscles were not fully stretched to body length while kept in culture. Williams and Goldspink (1978) have shown in the mouse soleus that immobilisation in the shortened position leads to a reduction in sarcomere number and an increase in sarcomere length. To resolve these questions it would be interesting to measure sarcomere number, and sarcomere length, in amphibian muscles cultured under stretched and unstretched conditions.

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THE ROLE OF JUVENILE HORMONES IN THE LARVAL DIAPAUSE OF THE CRICKET *GRYLLUS CAMPESTRIS* (ORTHOPTERA)

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Abstract—Titration of juvenile hormones in diapausing and non-diapausing cricket of the species *Gryllus campestris*, juvenile hormone 1 injections into diapausing animals which break the diapause, and titration of ecdysteroids in these injected animals demonstrate that the diapause in this species is characterized by a lack, and its termination by a synthesis, of juvenile hormones.

INTRODUCTION

Gryllus campestris is a univoltine insect which enters diapause during the penultimate larval instar (=protostage). This diapause is induced by high temperature (30°C) during the three last instars before the protostage. If the speed of development is slowed by low temperature (20°C) during the same period (three last instars) before the protostage, the diapause does not appear (Ismail and Fuzeau-Braesch, 1976a). The photoperiod is effective only in counting the number of instars and in synchronizing the protostage moulting on entering diapause. Once the diapause is induced it is completed by exposing the animals to cold (12°C) for 12 weeks, then transferring them to high temperature (30°C) for moulting. Complete development then takes 13–14 days.

From the physiological point of view, this diapause is controlled by the brain (Ismail and Fuzeau-Braesch, 1983). The hormonal level of ecdysteroids in the diapausing protostage remains very low during the whole instar (Ismail and Fuzeau-Braesch, 1979). Injection of 20-hydroxyecdysone does not eliminate diapause and the metabolic fate of ecdysone is excreted during diapause as inert material (Ismail and Fuzeau-Braesch, 1976b). In the course of a preliminary assay (Ismail and Fuzeau-Braesch, 1983), juvenile hormones 1, 2 and 3 were injected into the diapausing protoinstar. The diapause was reduced to a great extent and JH 1 was more effective than the other two hormones.

The aim of the present work is to investigate the level of juvenile hormones in diapausing and non-diapausing animals and to specify the role played by JH 1 in the diapausing state.

MATERIALS AND METHODS

Adults were collected in May (Gif sur Yvette, Essonne). Eggs were laid in laboratory and the animals were bred under optimal conditions adopted for the induction of diapause: 30°C and 10 hr light per day, or conditions which produce nondiapausing or hibernating insects as previously described (Ismail and Fuzeau-Braesch, 1976a). All crickets used in the experiments were of the first or second generation.

Juvenile hormone titre

Experimental animals. Diapausing animals hibernating at 12°C: the insects were reared at 30°C before the protostage, transferred in the protostage to cool condition (12°C) for 12 weeks, then transferred to 30°C for moulting. The samples were carried out on weeks 3, 6, 9 and 12 at 12°C (=series 1), and for 13 days after transferring at 30°C (=series 2).

Diapausing animals (series 3): animals were reared at 30°C before the protostage and maintained in the same conditions during this instar. The samples were carried out on days 1, 5, 10, 20, 30 and 45 of the protoinstar.

Nondiapausing animals (series 4): animals were reared at 20°C before the protostage, then were transferred to 30°C in the protostage for moulting which produced a direct development. Samples were carried out for 13 days at 30°C, this time being necessary for the moulting cycle.

Haemolymph collection. Haemolymph (200 μl) was collected for each sample from 20–40 protostage larvae in different experimental conditions, and placed immediately in 1 ml of methanol/diethyl ether (1:1 v/v). The antennae of each animal were cut down to the base. Then the living animal was put in a conical centrifuging tube with the head in the narrow part. Centrifugation was carried out at 4°C for 3–5 min at 1500 revs/min. The haemolymph obtained was clear.

RIA assays for JHs. A known quantity of tritiated JH 1 (5000 dis/min JH 1 ^{3}H, NEN, 10 Ci/mM) was added to the haemolymph sample in methanol/diethylether. JH were extracted twice from methanol/diethylether with 4 ml of hexane. The upper phase contains the JH. The purifications were made as already described (Baehr *et al.*, 1981) by chromatography on silica column plus chromatography on Sep Pak (C 18 Waters) column and reverse-phase liquid chromatography (μCN Waters columns; solvent: methanol/water 4/6; flow rate 1 ml/min). Fractions of 0.8 ml from 1 to 30 were collected. The immunoreactivity of every fraction was assayed with JH 1 RIA, JH 2 RIA and JH 3 RIA directly in the solvent (methanol/water 4/6). A sample containing 5 ng of JH 1 plus 5 ng JH 2 and 5 ng of JH 3 was assayed as the biological sample; this enabled us to know the retention time of the JHs, to calculate the cross-reactivities and to check for solvent blanks. In the HPLC system used, JH 1 was collected in fractions 16–17 and 18, JH 2 in fractions 13 and 14 and JH 3 in fractions 11 and 12.

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The *Gryllus* haemolymph contains immunoreactive substances eluted in fractions corresponding to JH 1, JH 2 and JH 3 and also in fractions 1–10 which were not correlated with JHs. Moreover, these non-JHs immunoreactive substances fluctuated quantitatively from one sample to another (from 0 to 41‰). Fraction 13 contained an immunoreactive material slightly recognized by JH 2 antibodies, highly bound with JH 3 antibodies but none with JH 1 antibodies. This substance was not present in all samples. As it did not have the cross-reactivity expected for JH 2 and JH 3 RIAs we concluded it was not JH 2.

The immunoreactivity of the fractions containing JH 1 was correlated with the elution of the tritiated JH added at the beginning (dilution test) and is in agreement with the cross-reactivity in JH 2 and JH 3 RIAs.

The immunoreactivity of the fractions containing JH 3 was also in agreement with the cross-reactivity in the JH 1 and JH 2 RIAs. Because of the non-JHs immunoreactive substances, HPLC was needed for the purification and analysis of every sample. Only JH 1 and JH 3 immunoreactive substances were detected in the corresponding fractions. The recoveries were measured by the tritiated JH 1 added at the beginning. For JH 3, the data were corrected using the same recovery to that of JH 1.

Juvenile hormone injection

JH 1 (Sigma) was dissolved in 10 μl of corn oil. A dose of 3 μg of the hormone was injected daily for a successive 10 days. A strong dose of 10 μg of JH 1 was injected once, or every 2 days for a week (i.e. four times). Whatever the dose, the injection was always done on the first day of protostage.

Ecdysteroids titre under the influence of JH 1 injections

Experimental animals. Diapausing animals were kept at a temperature of 30°C conditions. JH 1 was injected under the most effective condition previously described, i.e. 10 μg four times a week (every 2 days).

Haemolymph collection. After the injections 10 μl of haemolymph were collected from cooled insects (12°C for 2 hr) by a prick in the trochanter of the posterior leg and directly put to congeal until titration. The collection of haemolymph began 4 hr after the last injection of JH 1 and then every day with new animals each time. All these insects were followed individually until their moulting, which enabled us to know *a posteriori* the physiological age of the animal related to the elimination of the diapause.

RIA assay for ecdysteroids. Ecdysteroids were extracted twice from haemolymph samples by 1 ml methanol. The methanolic phase was evaporated to dryness and the samples were redissolved in phosphate buffer (0.1 M, pH 7.4, 0.1% gelatine). Further dilutions were made in the same buffer for radio-immunological assay as previously described (Porcheron *et al.*, 1976, 1977). In this system 20 hydroxyecdysone was used as standard and the results are expressed in ng ecdysterone equivalent per ml of haemolymph. Cross-reaction between ecdysone and 20 hydroxyecdysone was 100‰ thus, the total amount of the two circulating hormonal was measured.

RESULTS

Juvenile hormones titre (Table 1)

The juvenile hormones remain undetectable during 3 and 6 weeks in diapausing larvae kept cool (12°C) in hibernating conditions (1). Then, in spite of staying in cool conditions, they exhibit detectable amounts of hormones at 9 (mean of JH 1: 3 ± 1.5 ng/ml, JH 3: 0.4 ± 0.1) and 12 weeks (mean of JH 1: 1.3 ± 0.8, JH 3: 0.2 ± 0.1 ng/ml), which are not significantly different from one another.

After transferring at 30°C (2), the same kind of animals show an appreciable amount of hormones (1.8 ± 0.5 JH 1 and 1.0 ± 0.2 ng/ml JH 3) during the first 6 days which is followed by a decrease to a very low level (0.2 ± 0.1 JH 1 and 0.3 ± 0.1 ng/ml JH 3) during day 7 to the moult. When the diapausing protostage is maintained in non-ecological conditions

Table 1. Levels of JH I and JH III of the protostage larvae of *Gryllus campestris* under different experimental conditions related with the diapause

Experimental conditions		Mean JH 1 level (ng/ml)	Mean JH 3 level (ng/ml)
(1)	Diapausing protostage hibernating at 12°C		
	week 3	0	0
	week 6	0	0
	week 9	3 ± 1.5	0.4 ± 0.1
	week 12	1.3 ± 0.8	0.2 ± 0.1
(2)	Diapausing protostage which have been submitted to 12°C for 12 weeks and transferred to 30°C		
	days 1–6	1.8 ± 0.5	1.0 ± 0.2
	days 7–13 (moult)	0.2 ± 0.1	0.3 ± 0.1
(3)	Diapausing protostage without cooling and maintained at 30°C		
	day 1	0	0
	day 5	0	0
	day 10	0	0
	day 20	0	0
	day 30	0	0
	day 45	0	0
(4)	Non diapausing protostage		
	days 1–6	0.4 ± 0.1	0.5 ± 0.1
	days 7–13 (moult)	0.2 ± 0.04	0.6 ± 0.1

No. of pooled samples between 11 and 21, except (1) four samples for each mean.

Table 2. Effect of injection of JH 1 on larval diapause of *Gryllus campestris*

Dose	No. of injections	No. of larvae	% of larvae without diapause	Mean duration of protostage in days ± SEM
JH 1				
3 µg	10 successive days	15	82	26 ± 4.4
10 µg	1 time, first day	25	33	39 ± 4.3
10 µg	4 times for a week every 2 days	108	98	12 ± 1.95
Corn oil				
5 µl	1 time, first day	15	0	54 ± 3.2
5 µl	4 times for a week every 2 days	15	15	44 ± 3.5
	Control	?	6	52 ± 2.3

at a high temperature (30°C) without cooling (3), for 45 days, there is no moult and no detectable hormones at all. If the diapause has not been induced by the described treatment (4), the protostage is characterized by a detectable but low level of hormones: days 1–6, 0.4 ± 0.1 JH 1 and 0.5 ± 0.1 JH 3 ng/ml; day 7 to the moult, 0.2 ± 0.04 JH 1 and 0.6 ± 0.1 ng/ml JH 3. JH 2 is never detectable in this instar and under these conditions.

Injections of JH 1 on larval diapause (Table 2)

When a small dose of 3 µg of JH 1 is injected daily for 10 days (total 30 µg), 82% of the larvae are without diapause with an average duration of the instar of 26 ± 4.4 days. A larger dose of 10 µg injected only once, on the first day after moulting of the protostage, gives a slight result with 33% of nondiapausing animals and a mean duration of the instar of 39 ± 4.3 days. When the same dose of 10 µg is injected 4 times a week, every 2 days (total 40 µg), the diapause is totally eliminated (98%) and the average duration of the protostage decreases to 12 ± 1.95 days. All the adults coming from JH 1 injected protostage and whose diapause elimination occurs successfully, show a noticeable shortening of the wings whereas the normal morphology of this species is long wings.

The two different controls with corn oil and the control without any injection exhibit approx. the same results with a large diapause and an average duration of the instar of 44–54 days.

If ecdysterone is injected together with JH 1, whatever the dose of JH 1, we observe a high mortality rate (30–70%) and consequently these kinds of experiment (results not shown) are discontinued.

Ecdysteroids titre after injections of JH 1 (Table 3)

Since JH 1 injected to the protostage, whose diapause was induced by high temperature conditions as previously described, is able to break this diapause, levels of ecdysteroids have been followed in these animals, in comparison with controls which were submitted to the same conditions but without JH 1.

After a week of injections, diapausing protostages show a slight increase in the amount of ecdysteroids from days 7–6 (460 ± 201 and 839 ± 367 ng/ml) and a very high increase for days 4–2 before the moult (1315–3855 ± 3 ng/ml). When haemolymph is collected one day or less before the moult, the quantity of the hormone is very low (128 ± 87 and 41 ± 17 ng/ml) (1).

If we compare these results with those obtained with non-diapausing protostages (2), we observe that the peak of ecdysteroids is very similar in level (maximum: 3750 ± 350 ng/ml) and in timing (increase between days 5–9).

On the contrary, the diapausing protostage maintained at 30°C without hibernation cooling and with control injections of corn oil is characterized by a poor and approximately constant ecdysteroids content during the same period (between 99 and 211 ng/ml with slight fluctuations without apparent signification).

DISCUSSION

Juvenile hormones JH 1 and 3 are synthesized at the end of hibernation by diapausing animals and this corresponds to ecological conditions. JH are secreted before heating, that is to say during the end of hibernation just before spring for which the insects are physiologically early. The level of JH remains the same when the insects are heated after the hibernation treatment; then JH content fails and moulting occurs. If diapausing crickets are maintained in hot (i.e. non-ecological) conditions, they do not synthesize any JH which leads to a very long period without moult. Thus, we can conclude that JH synthesis corresponds to the switch to a normal development after diapause.

The following experiment which consists of injecting JH 1 into diapausing animals maintained at high temperatures without cooling leads to a break of the diapause and thus confirms that JH controls the termination of this state, whereas injection of 20 hydroxyecdysone was ineffective as previously described (Ismail and Fuzeau-Braesch, 1976b).

The observation of the JH amounts in non-diapausing insects show surprisingly that, for this direct development, the level of JH is not so high as in the termination of the diapause; it stays at medium level with some fluctuating values which may be perhaps related to JH esterase activity (Bean *et al.*, 1982). Thus, higher levels of JH are required for terminating the diapause after hibernation.

Finally, the titration of ecdysteroids in diapausing animals injected with JH 1 shows that the moulting hormone is synthesized slowly after a week of injection; then the amount exhibits an increase leading to the mould which is similar to those obtained in normal nondiapausing animals, which may exclude the possibility that JH lowers the cellular requirements for ecdysteroids (Sehnal, 1981).

However, we must emphasize that a difference occurs between the moult of nondiapausing animals and the moult of diapausing ones whose diapause

Table 3. Levels of ecdysteroids in the protostage of *Gryllus campestris* under different experimental conditions: influence of JH 1 injections on the secretion of ecdysteroids

Experimental conditions	Ecdysteroids in ng/ml ± SEM
(1) Diapausing protostages at 30°C; after a week injections (10 μg × 4), days before next moulting	
7	460 ± 201
6	839 ± 367
5	324 ± 54
4	1315 ± 42
3	3855 ± 3
2	3785 ± 901
1	128 ± 97
< 1	41 ± 17
(2) Non-diapausing protostage at 30°C*	
Day	
1	110 ± 3
2	180 ± 10
4	105 ± 4
5	1500 ± 350
6	2600 ± 200
7	2000 ± 300
8	3750 ± 350
9	1650 ± 350
10	160 ± 30
(3) Diapausing protostage maintained at 30°C injected with corn oil; days after injections	
Day	
1	150 ± 49
2	99 ± 21
3	99 ± 13
4	68 ± 10
5	211 ± 20
6	158 ± 28
8	144 ± 24
9	102 ± 16

*See Ismail and Fuzeau-Braesch, 1979.

was broken with JH injections; all the adults obtained have short instead of long wings, this means that the timing of events which is artificially disrupted in the experiment has a morphological consequence, eventually related to the target organs which are slowly impregnated by JH during the end of hibernation as seen above, or with the high level of injected exogenous JH 1.

The control of the termination of diapause by an increase of JH is in agreement with the results obtained in other species (Schooneveld *et al.*, 1977; Bhaskaran *et al.*, 1980; Bradfield and Denlinger, 1980; Walker and Denlinger, 1980). On the contrary, our results differ from those of Yin and Chippendale (1979) and Mane and Chippendale (1981) in *Diatraea grandiosella* where high JH levels is related to the maintenance of the diapausal arrest of development. The diapause related to the absence of JH and ecdysteroids is primarily controlled by inactivity of corpora allata and brain as shown by brain implantation experiments (Sellier, 1955; Ismail and Fuzeau-Braesch, 1976b).

In this species, on the one hand, injection of 20 hydroxyecdysone remains without effect (Ismail and Fuzeau-Braesch, 1976b), on the other hand, ecdysteroids production follows very slowly the injections of JH 1; thus we are led to think that the activator role of JH may not be only a direct prothoracotropic effect as described by Cymboroski and Stolarz (1979) and Hiruma (1980, 1982) in other species.

As demonstrated by other authors (Thomsen and Lea, 1968; McCaffery and Highnam, 1975), the corpora allata and JH may be directly active in the synthesis and release of neurosecretory materials in the brain of insects, as may be the case in our species (Ismail and Fuzeau-Braesch, 1983).

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EFFECT OF DIETARY PURINES AND PYRIMIDINES ON GROWTH AND DEVELOPMENT OF *DROSOPHILA**

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Abstract—1. Addition of purines (adenine and guanine) and pyrimidines (cytosine and thymine) to the nutrient medium affected the growth and development of *Drosophila melanogaster*.

2. Adenine was detrimental to the metamorphosis of the flies and caused weight reduction in newly hatched flies.

3. Addition of 0.23% of adenine per 100 g of the nutrient medium was sufficient to reduce by one-half the number of adult flies hatched ($LD_{50} = 0.23\%$).

4. Adenine also changed purine–pyrimidine profiles in the larval and pupal stages, but had no effect on these profiles in adult flies.

INTRODUCTION

Drosophila melanogaster has been widely used in heredity studies (Sinclair *et al.*, 1981; Pelliccia and Sofer, 1982). The short life cycle, from egg-hatching to emergence of the imago, is completed in about 10 days at 25°C. Furthermore, a large genetically homogeneous progeny can be derived from single-pair matings. Several studies have also illustrated the value of *Drosophila* as an experimental model for evaluating the biological influence of environmental factors, such as diet (Vitt *et al.*, 1982), insecticide (Morton and Singh, 1982), naturally occurring mutagens (Clark, 1982), and temperature (Tantawy and Mallah, 1961).

Exogenous, chemically and physically similar purines have been shown to have very different biological effects in rats (Savaiano and Clifford, 1978), chickens (Hevia and Clifford), and humans (Clifford *et al.*, 1976). Among the purines, adenine, guanine, hypoxanthine, xanthine, only adenine is absorbed as such from the gut of rats (Savaiano *et al.*, 1980) and produces a marked hyperuricemia in humans (Clifford *et al.*, 1976). Since there are no reports on the effects of exogenous purines on reproduction in any species, the present study was undertaken to study the effects of adenine, guanine, cytosine and thymine on reproduction and growth of *Drosophila melanogaster*.

MATERIALS AND METHODS

Pairs (1 male and 1 female) of *Drosophila melanogaster* yellow body mutant virgin flies were isolated from the Emporia State University, Division of Biological Science stock culture which was routinely maintained at 24°C on a cornmeal medium (Strickberger, 1962). In the first experiment, four pairs of virgin flies of both sexes were isolated (Strickberger, 1962) and placed into 200 ml glass milk bottles containing about 75 g of the control diet which was a blended mixture of the following ingredients: 935 ml water, 7.9 g agar, 47 ml malt, 95 ml Karo syrup, 125 ml 30% ethanol, 14 g yeast, 75 g cornmeal, 3 ml antibacterial (Zephiran) and 3.7 ml antimycotic (Tegosept-M). The antimicrobial and antimycotic agents are non-toxic to flies. Additional pairs of flies were also placed in similar bottles containing the same diet supplemented with 0.4% adenine, guanine, cytosine and thymine (Sigma Chemical Co., St Louis, MO), individually. The flies were grown and mated under a controlled temperature of 24°C and an environment with a 12 hr light and a 12 hr dark cycle. There were four replicate bottles for each dietary treatment. Seven days were allowed for the parental flies to lay their eggs, the flies were then removed from the bottle and discarded. The adult flies emerging from the puparium were counted, weighed and stored at −70°C for later purine and pyrimidine analysis. The adult flies were anesthetized with diethyl ether to facilitate weighing.

In the second experiment, increasing levels (0, 0.1, 0.15, 0.2 and 0.3%) of adenine were added to the control diet which was then placed in 50-ml glass bottles with cotton stoppers. Each level of adenine was replicated 10 times for a total of 50 bottles. One pair of virgin flies was added to each bottle and removed after 7 days. The bottles were maintained in the same environmental conditions as experiment 1. Larva, pupa and newly hatched adults were harvested, counted, washed 3 times with buffered saline (Krebs and Hanseleit, 1935), and stored at −70°C until analyzed.

Samples for purine and pyrimidine analysis were dried to a constant weight at 105°C, homogenized in 40 vol of 0.6 M perchloric acid (PCA), and heated in a boiling water bath for 1 hr. The samples were cooled, centrifuged at 14,000 *g* for 15 min at room temperature and the supernantant clarified by filtration through 0.45 μ filter. The excess PCA in the clarified supernatant was removed with an equal volume of a freon–octylamine mixture (Kkym, 1975). The upper phase was removed and diluted with an equal volume of 5 mM $NH_4H_2PO_4$ buffer, pH 2.85 and a 10 μl aliquot was applied to the high pressure liquid chromatography (HPLC) column.

The liquid chromatograph was a Varian LC 5060 (Palo Alto, CA) with a 4.6 × 300 mm, weak anion exchange column (MicroPak AX-5) with a 5 μ dia particle size and a diamine functional group. Column effluent was monitored at 254 nm using a Varichrom-5 u.v. detector and recorded on a Varian strip chart recorder (4.25 mm/min). The liquid chromatograph was connected in series with low dead volume stainless steel tubing to a Hewlett–Packard 8450 spectrophotometer (Palo Alto, CA) having an 8 μl flow cell to permit spectrophotometric scanning of peaks as they emerged from the detector flow cell.

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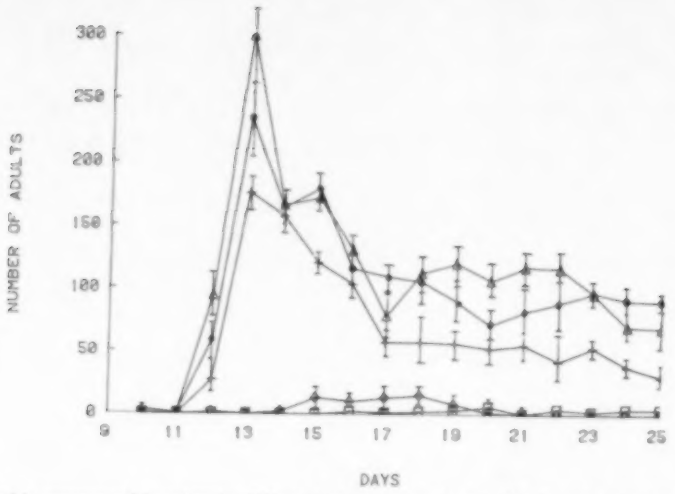

Fig. 1. Control is represented by triangles. Effect of adenine ◇—◇, guanine ●—●, cytosine +—+, and thymine □—□ on the number of flies hatched. Each point represents the offspring of 4 bottles, each bottle originally contained 4 pairs of flies.

Freshly prepared aqueous standard solution containing a 5 mM mixture of each of the following purines and pyrimidines: uracil, uridine, thymine, adenine, guanine, hypoxanthine, inosine, guanosine, cytosine, cytidine, was applied to the HPLC column. The various purines and pyrimidines were separated using the following buffers: buffer A, 870 ml of acetonitrile plus 130 ml of 5 mM $NH_4H_2PO_4$ (pH 2.85); buffer B, 5 mM $NH_4H_2PO_4$ (pH 2.85); and buffer C, 750 mM $NH_4H_2PO_4$ (pH 4.5). The elution program consisted of 100% buffer A for 15 min followed by a gradient of buffer B from 0 to 100% at 4%/min. This was followed by a gradient of buffer C from 0 to 100% at 2.5%/min. The flow rate was 0.4 ml/min for the first 15 min, then was increased to 1.0 ml/min during the next 2 min and remained at this rate throughout the analysis. At these flow rates, the column pressure ranged from 20 to 70 atm. The column was maintained at 34°C. The chemical identity of the peaks eluted from the HPLC was confirmed by retention times and by their characteristic u.v. spectra at basic, neutral and alkaline pH.

The number of newly hatched larva to emerge as adults from bottles containing media with increasing levels of adenine (experiment 2) were counted each day beginning on day 10. The numbers hatched on the peak day of emergence were plotted against the concentration of adenine in the medium in order to determine the adenine level which reduced the number of hatched insects by 50% (Bliss, 1935).

Because the larvae were actively moving in the diet, it was removed from the bottles and was broken apart into small particles with sterilized buffered saline and the larvae were collected and stored at −70°C until analyzed for purines and pyrimidines. No attempt was made to separate the larvae into respective instars.

RESULTS

The largest numbers of hatchings occurred on day 13 in all dietary groups. Dietary purines and pyrimidines had very different effects on the 13th day peak reproduction as well as on the total cumulative reproduction as shown in Figs 1 and 2. Guanine and cytosine reduced the number of offspring by 30 and

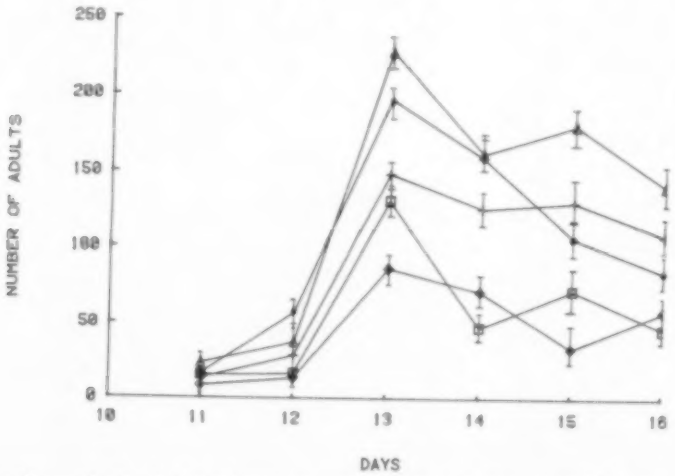

Fig. 2. Effect of increasing levels of adenine on the number of flies. C = triangle. 0.1% = ●—●; 0.15% = +—+; 0.2% = □—□; 0.3% = ◇—◇. Each point represents the offspring of 10 bottles, each bottle originally contained 1 pair of flies.

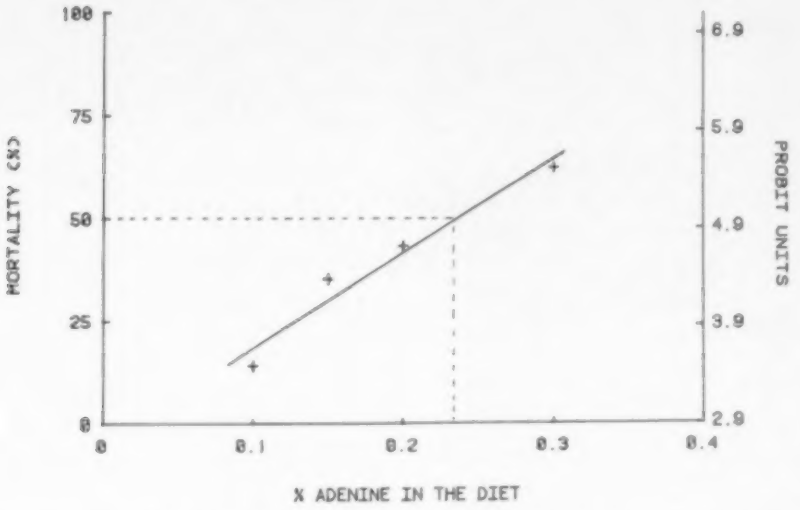

Fig. 3. Dose–response relationship of adenine and the number of flies hatched used in calculating the level of adenine needed to reduce the number of hatched flies by 50%. Each point represents the offspring of 10 bottles, each bottle contained 1 pair of flies.

50%, respectively, compared with controls (Fig. 1). Adenine and thymine, on the other hand, completely prevented hatching.

The inhibitory effect of dietary adenine on reproduction was dose dependent (Fig. 2). Hatchings decreased as the level of adenine in the diet increased from 0.1 to 0.3%. When the number of adult flies hatched was plotted, on the probit scale (Bliss, 1935), against the level of dietary adenine, it was calculated that 0.23% adenine caused a 50% reduction in the number of flies hatched at the 13 day peak hatch period (Fig. 3).

The dietary purines and pyrimidines also affected the body weight of newly-hatched day-old flies (Fig. 4). The reduction in body weight was approximately 50, 10 and 5% for adenine, guanine and cytosine, respectively. Thymine did not affect body weight as compared with the control.

Adenine affected the purine profiles of the 3 developmental stages in different ways. Adenine increased the levels of hypoxanthine and inosine in larva (Fig. 5), and the levels of uridine, hypoxanthine, and adenine in pupa (Fig. 6). Adenine reduced the level of adenine in larva and had no effect on that of the adult (Fig. 7).

There were two significant qualitative differences in the purine profiles among the 3 stages of insect. The first difference was a large peak preceding the thymine, which was present in larva (Fig. 5) and pupa (Fig. 6) but absent in adults (Fig. 7). The second difference was a

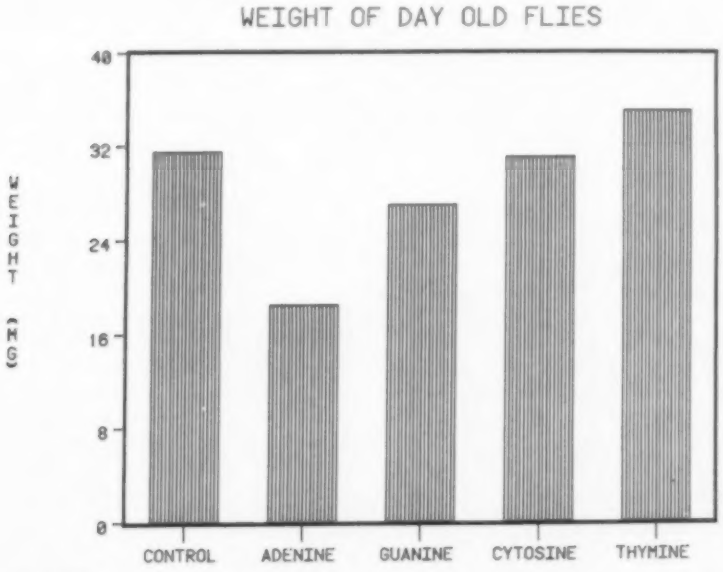

Fig. 4. Effect of purines and pyrimidines on body weight of 50 newly hatched day old flies.

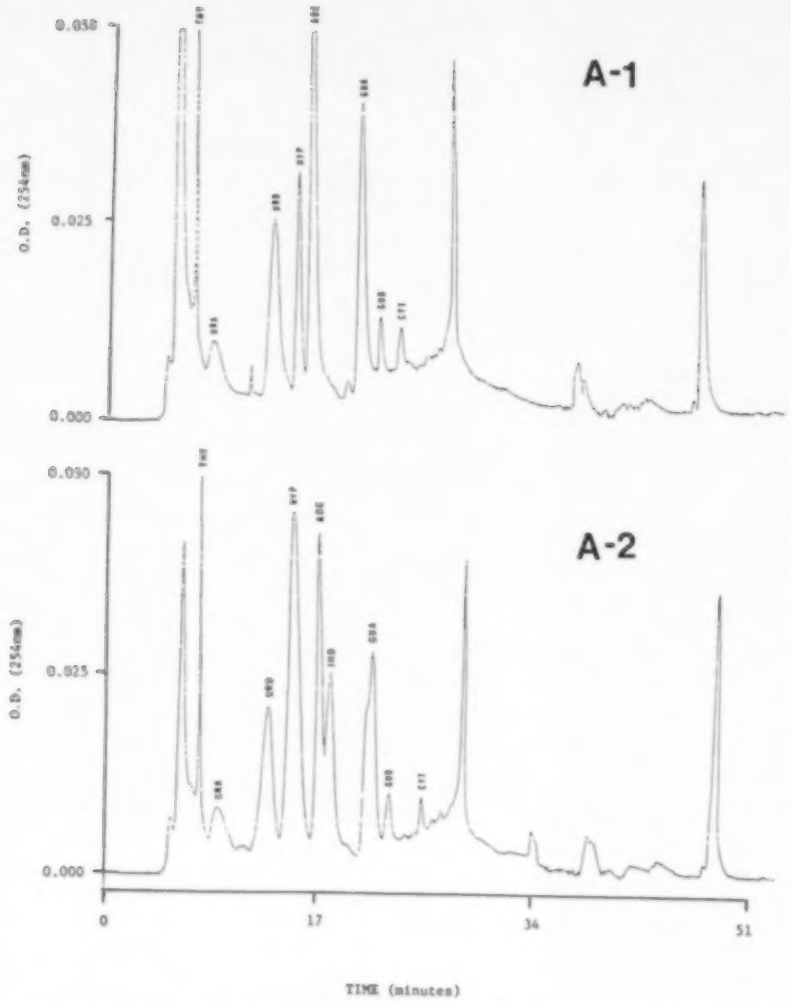

Fig. 5. Purine and pyrimidine profile of control (A-1) and adenine (0.15%) fed larva (A-2). THY = thymine; URA = uracil; URD = uridine; HYP = hypoxanthine; ADE = adenine; INO = inosine; GUA = guanine; GUO = guanosine; CYT = cytidine.

peak eluting at 50 min which was present in larva and adults but absent in pupa. These peaks presently remain unidentified.

DISCUSSION

Unlike the other purine and pyrimidine bases, adenine and thymine depressed the number of flies hatched, and adenine caused weight reduction in newly hatched flies. Three possibilities may account for the detrimental effects of adenine in these experiments. Firstly, the ovulation by parental flies might have been impaired due to the ingestion of adenine. Secondly, the development of the larva may have been inhibited due to the ingestion of adenine. Thirdly, the differences in the effects of the bases may be due to changes in pH of the supplemented food in comparison with the control food. The pH of all five diets in experiment 1 was 4.5. We concluded that the effects of adenine were not due to a change in pH. Parental ovulation and larvae development could be due to perturbations of adenine metabolism at the cellular level.

The reduced rate of weight gain of flies fed adenine indicates that adenine in the medium had a harmful effect on metamorphosis. However, the effect of the other supplements on the mortality rate did not seem to be correlated with the weight of the flies which emerged. The bases which were detrimental to fly weight were the adenine and guanine. The pyrimidines, cytosine and thymine, did not affect body weight as significantly. Thus, our data suggested that the supplementation of purines and pyrimidines in the diet mediates different physiological effects on the metamorphosis of *Drosophila melanogaster* and supports the second hypothesis above that larval development was inhibited by adenine.

Since the lower concentrations of adenine reduced the number of larvae, and hence adults, arising from a given number of eggs (Crammer and Cummings,

unpublished data), the larvae sampled for HPLC analysis may represent two populations. Larvae may represent those that are genetically resistant and those that are sensitive to adenine. Those that are resistant would survive and give rise to resistant adults while nonresistant larvae would die in either a later instar or pupal stage. Hence, the only adults present were those resistant to adenine. This may explain why adults had the same purine–pyrimidine profile in the presence and absence of adenine (Fig. 7) yet larvae and pupae show different profiles (Figs. 5 and 6).

These same data also seem to support the conclusion that adenine feeding within a short period did not have an affect on fecundity of the parents since larvae and pupae hatched in the presence of adenine have HPLC profiles which are different from those of controls. Although experiments are in progress to evaluate fecundity further, the HPLC profiles imply that ingestion of adenine, the second hypothesis proposed, is the most likely cause of mortality in the fruit fly.

The bases which had the most detrimental effect on the number of flies emerging were adenine and thymine. Adenine is the purine which binds with thymine, a pyrimidine, to form the appropriate base pair for DNA complimentarity (Watson and Crick, 1953). Eurcaryotes are also known to use up to 200 adenine residues to form a 3-poly (A) tail on m-RNA which is necessary for translation (Darnell, 1982). Furthermore, adenine also reduces the thymidine toxicity in cultured cells (Leyva *et al.*, 1982). Additional studies are necessary to determine whether or not thymidine produces a beneficial effect in the development of the flies fed with a diet supplemented with adenine since KC3-H *Drosophila* cell cultures exhibit no adenine toxicity in the presence of thymine (Guanipa and Sobieski, unpublished data).

Alterations in purine catabolism are associated with immunodeficiency diseases in humans (Seegmiller, 1978). The present data are consistent with other studies which have shown that adenine compared to other purines is more toxic in a variety of other species,

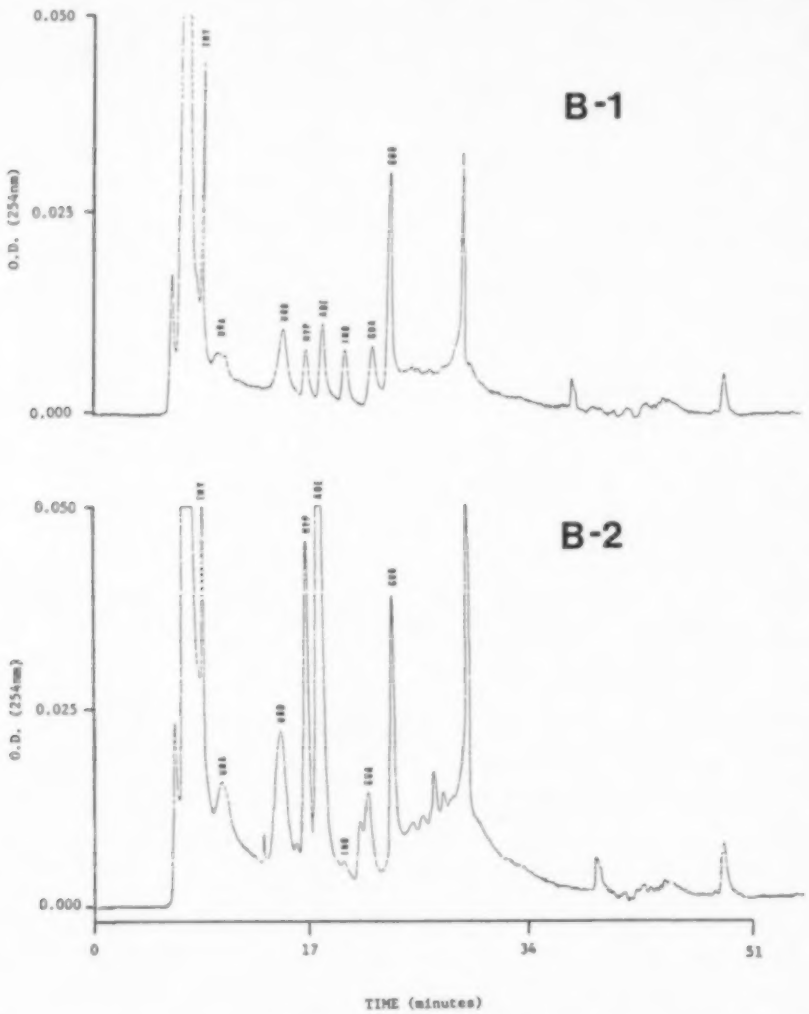

Fig. 6. Purine and pyrimidine profiles of control (B-1) and adenine (0.15%) fed pupa (B-2). Abbreviations same as in Fig. 5.

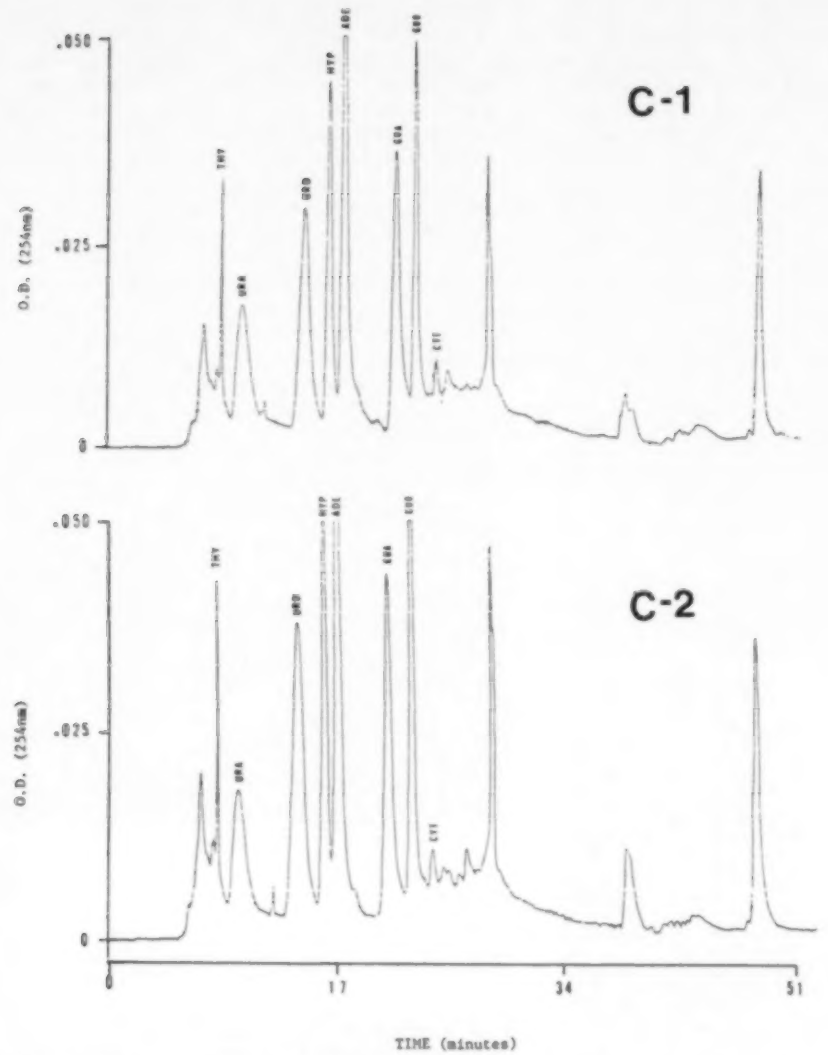

Fig. 7. Purine and pyrimidine profiles of control (C-1) and adenine (0.15%) fed adults (C-2). Abbreviations same as in Fig. 5.

including the rat, chick, humans and bacteria (Levine and Taylor, 1982). Adenine is also more toxic than guanine, hypoxanthine or xanthine when added to cell lines of *Drosophila* in culture (Guanipa and Sobieski, unpublished data). Adenine and hypoxanthine have also been shown to inhibit purine reutilization by chicken hepatocytes (Clifford and Clifford, 1981). The level of adenine in the nutrient medium needed to reduce by 50% (LD_{50}) the number of adult flies hatched was 0.23 g/100 g. The consistency of the adenine effect in various whole animals and in their cell lines supports the universality of this effect at the cellular level.

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REVIEW

SUGAR AND AMINO ACID TRANSPORT IN FISH INTESTINE

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Abstract—1. Morphological properties of fish intestines vary with diet. Carnivores have short guts with highly elaborate mucosal folding in the upper intestines; herbivores have long guts which appear structurally uniform from stomach to rectum.

2. Brush border membranes of many fish intestines display at least two transport processes for each organic solute, one an Na^+-dependent, saturable carrier mechanism, and the other a non-saturable influx pathway which may be simple diffusion.

3. Intestinal epithelial cells from freshwater fish can accumulate nutrients to concentrations in excess of those in the gut lumen; those of marine fish can not.

4. Net transepithelial nutrient transport in upper intestine is greater in freshwater fish than in marine forms as a result of considerable solute backflux from epithelium to lumen in the latter.

5. In many fish the lower intestine displays a significant net transmural flux of nutrients that may contribute to total organic solute absorption.

6. Intestines of freshwater fish have a serosa positive (relative to mucosa) electrical potential difference; marine fish display a negative serosa.

7. Addition of organic solutes to intestines of freshwater fish hyperpolarizes the electrically positive serosa; in marine forms a depolarization of the serosa negative potential occurs. In both cases this appears due to increased net transmural sodium transport coupled to net nutrient flow.

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INTRODUCTION

The aim of this review is to collect and assess much of the available information published mainly over the last two decades on sugar and amino acid transport in fish intestines and to compare the nutrient transfer mechanisms in these lower vertebrates with those described in detail for mammals and other higher vertebrates. This review begins with a short anatomical description of fish gut morphology, comparing histological structures in carnivorous and herbivorous species. A discussion of papers characterizing solute influx, or initial entry rate of sugars and amino acids into fish absorptive cells from the luminal solution, follows. Then processes involved in solute accumulation by intestinal epithelial cells at steady-state will be examined. Other investigations characterizing the properties of transepithelial solute movements from one gut surface to the other will then be discussed. Finally, a review of papers concerning ionic and electrical properties of the fish intestine and how the presence of luminal sugars and amino acids affect these parameters will be given.

COMPARATIVE MORPHOLOGY OF FISH GUT

While mammalian intestine is clearly differentiated into distinct anatomical regions comprising the small (duodenum, jejunum, ileum) and large (colon) intestines, fish gut generally does not show such clear divisions and is often described only by the location of a particular segment relative to a fixed marker such as the stomach or rectum. A few generalities may be made about the anatomy of fish intestine that appear to have functional relevance.

The length of intestine in fish is a highly variable character and appears related to food habits (Bond, 1979). While carnivorous fish species possess short intestines, often a simple straight tube connecting stomach and rectum, herbivorous animals can exhibit an extremely long gut which coils extensively throughout the body cavity (Ferraris and Ahearn, 1983). Kapoor *et al.* (1975) compared the relative gut length (RGL: gut length/total body length) to the type of feeding habits in 50 fish species of the families Characinoidae and Cyprinidae and reported a gradual increase in RGL from strictly carnivorous to strictly herbivorous species; omnivores were intermediate in RGL. Reifel and Travill (1978) measured the RGL of ten species representing five teleostean families and various feeding habits, and confirmed earlier findings that omnivores had higher RGL values than carnivores. Such differences in gut length have marked effects on food retention time and perhaps on absorptive efficiency.

The general anatomical structure of fish intestinal wall is similar to that exhibited by higher vertebrate intestine. An absorptive epithelium faces the luminal contents and is responsible for transport processes involving nutrients, ions and water. In contrast to that of mammals, fish intestinal epithelium is not

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divided into villus and crypt regions (Kapoor *et al.*, 1975; Field *et al.*, 1978), but instead only has a single layer of columnar cells thrown into a series of longitudinal ridges. The epithelial cells are lined with a brush border, and in some primitive species may be ciliated (Mohsin, 1962). Ciliated columnar cells can be found in fishes with a poorly developed muscular layer where the cells aid in food movement (Patt and Patt, 1969). Goblet, as well as PAS and alcian blue-stained cells, are common in fish epithelium, and mucus can sometimes be seen lining the apical border of the cells (Farraris, unpublished observations). It is not yet established whether solute absorptive and secretory processes in fish gut occur in separate cell types within this epithelium.

Striking differences in the extent of epithelial folding occur between fish species and may relate to the amount of roughage passing through the intestine (Kapoor *et al.*, 1975), or to feeding habits and RGL (Ferraris, 1982). In carnivorous fish species, pos-

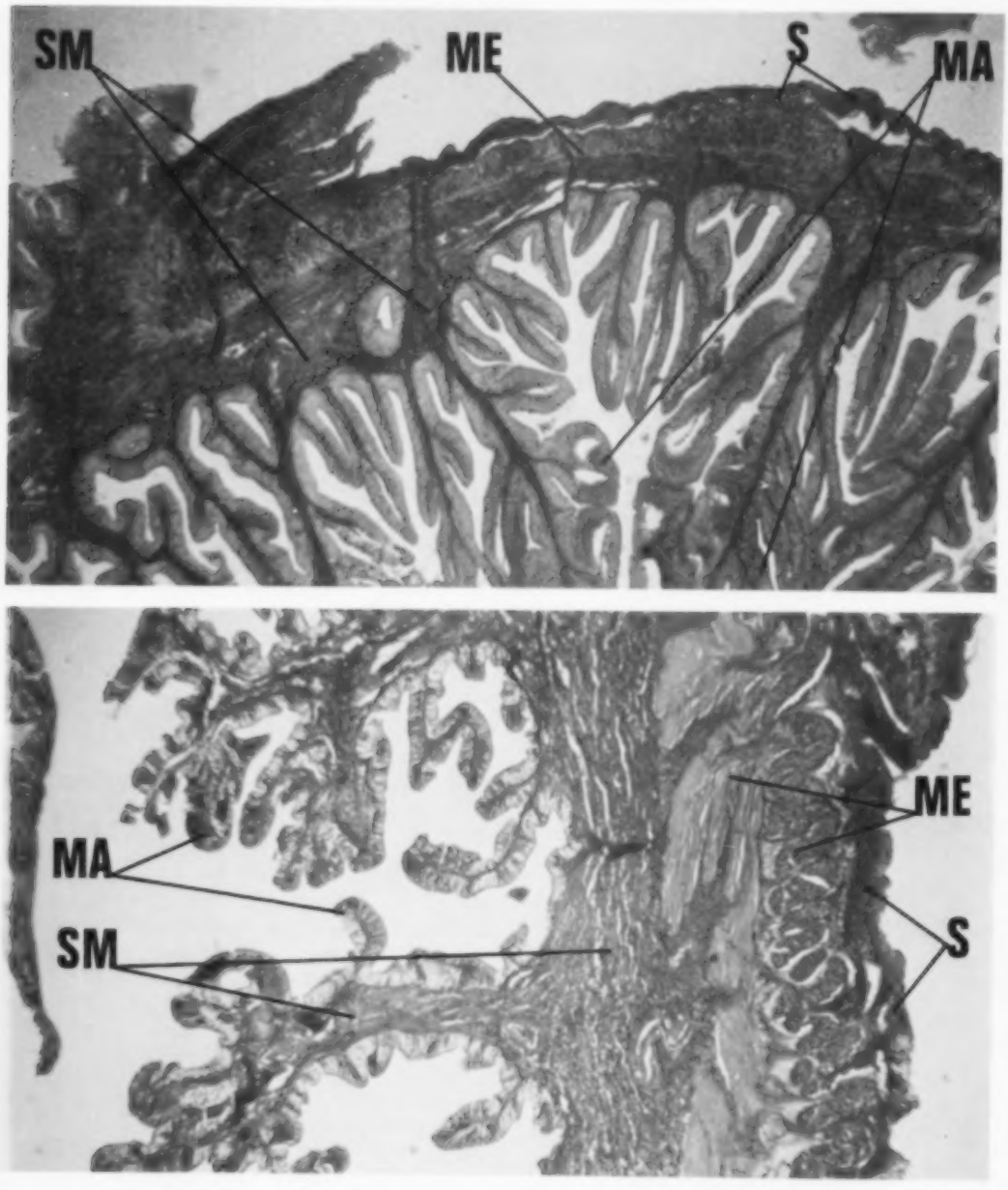

Fig. 1. Histological cross sections of carnivorous eel (*Gymnothorax undulatus*) upper and lower intestines. Top photograph: upper intestine illustrating highly branched submucosa (SM) and extensive elaboration of the absorptive mucosal (MA) epithelium. Muscle (ME) layers and serosal (S) lining are thin in this region and generally similar to these structures in higher vertebrates. Lower photograph: lower intestine displaying a reduced mucosal surface area and thickened submucosa and muscle layers compared to the more orally located portion of the gut. 34 × magnification.

sessing a short gut, the upper intestine (that portion closest to the stomach) exhibits extensive mucosal folding (Fig. 1, upper) thereby increasing the potential absorptive surface area for a given intestinal length. In this region of the gut the mucosa consists of an absorptive columnar cell type and goblet cells, the latter probably secreting mucus. In carnivorous fishes the lower intestine also exhibits an absorptive epithelium, but the folding in this location is considerably less developed than that of the upper gut (Fig. 1, lower). The hindgut of many species often displays an increase in the number of goblet cells and thickness of the muscularis. In both portions of the carnivore intestine the submucosa, muscle layers, and serosal lining are similar to these tissues in higher vertebrates. The long herbivore intestine exhibits less

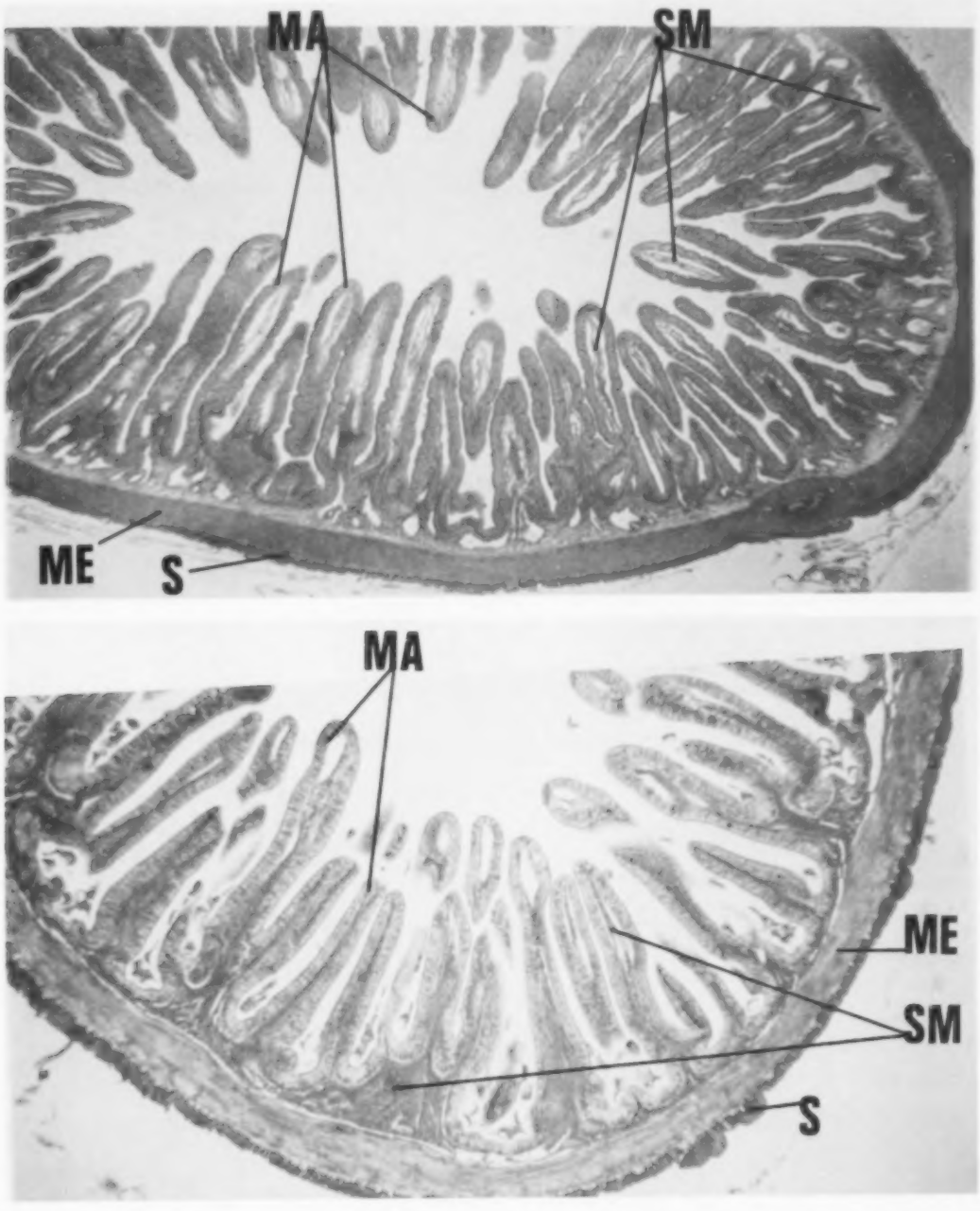

Fig. 2. Histological cross sections of herbivorous surgeonfish (*Acanthurus mata*) upper (top photograph) and lower (bottom photograph) intestines showing reduced mucosal absorptive surface area in both regions compared to the upper intestine of the carnivore (Fig. 1, top photograph). In addition, the herbivore gut exhibits a general structural similarity along its entire length. Abbreviations as in Fig. 1. 34× magnification.

epithelial folding along its extent than does the carnivore gut, and the general anatomical structures of upper and lower intestines in the herbivore appear remarkably similar (Fig. 2, upper and lower). These differences in epithelial histology between the two fish groups suggest that gut length and mucosal folding may be alternative strategies for maximizing absorption.

CHARACTERISTICS OF SUGAR AND AMINO ACID INFLUX IN FISH GUT

Solute influx from the luminal solution to epithelial cell across the brush border membrane in fish intestine is measured by incubating the gut sample in a radiolabelled medium for short enough time intervals to ensure minimal backflux of the substrate, but long enough to accumulate sufficient nutrient to detect. When these criteria are met, solute accumulation is a linear function of incubation time and the slope of the curve can be used to estimate unidirectional transfer or influx, J^i_{mc}, of substrate i from mucosal solution (m) to epithelial cell (c). In fish intestine, the relationship between J^i_{mc} and luminal substrate concentration has been reported in some studies to be hyperbolic and follow the Michaelis–Menten relationship:

$$J^i_{mc} = \frac{J^i_{max}\,[S]}{K^i_t + [S]} \qquad (1)$$

where J^i_{max} is maximal solute influx, K^i_t is the substrate concentration resulting in 1/2 maximal transport, and [S] is the luminal nutrient concentration. In other investigations nutrient influx across the brush border membrane appeared more complex and was described as the sum of two simultaneous processes, one exhibiting saturation kinetics of the type described by equation (1) above, and the other a transfer component with a rate that increased in a linear manner with an elevation in luminal substrate concentration. This composite analysis of solute influx follows the equation:

$$J^i_{mc} = \frac{J^i_{max}\,[S]}{K^i_t + [S]} + K^i_d\,[S] \qquad (2)$$

where K^i_d [S] describes the magnitude of the linear transport component. The three influx kinetic constants K^i_t, J^i_{max} and K^i_d have been used in the literature to describe carrier transport and apparent diffusional transfer of organic solutes across the epithelial brush border in various regions of the fish intestine.

Comparison of influx kinetic constants

Table 1 summarizes those studies which provided estimates of affinity constants (K^i_t) for monosaccharide and amino acid transport in fish intestines, although not all of these investigations employed techniques which fulfilled the necessary assumptions for correct measurement of solute influx described above. Carlisky and Huang's (1962) study was one of the earliest with estimated kinetic constant values for glucose absorption in fishes. However, their estimations of K^i_t for glucose were based on rates of transepithelial sugar movements and therefore they cannot be regarded as true influx constants. Their study, however, was one of the first to examine the effects of temperature and inhibitors on nutrient transport in fishes. Huang and Rout (1967) compared transport rates between D- and L- isomers of monosaccharides and amino acids as well as examined the effects of temperature on their rates of transfer. These experiments also employed transepithelial transport rates and demonstrated concentration-dependent solute movements, but the kinetic constant estimates which the authors derived from these studies do not reflect the activities of the brush border membrane alone.

Table 1. Influx affinity constants of amino acids and sugars in isolated fish intestines

Fish	Diet	Organic solute	K^i_t (mM)	Reference
Sugars				
Spiny dogfish	Carnivore	Glucose	20	Carlinsky and Huang (1962)
Killifish	Omnivore	Galactose	3*	Huang and Rout (1967)
Tench	Omnivore	Methyl-glucoside	2	Cartier *et al.* (1979)†
Winter flounder	Carnivore	2-Deoxy-D-galactose	10*	Naftalin and Kleinzeller (1981)
Surgeonfish, upper intestine	Herbivore	Glucose	0.6	Ferraris and Ahearn (1983)†
Surgeonfish, lower intestine	Herbivore	Glucose	0.4	Ferraris and Ahearn (1983)†
Eel, upper intestine	Carnivore	Glucose	0.3	Ferraris and Ahearn (1983)†
Eel, lower intestine	Carnivore	Glucose	0.3	Ferraris and Ahearn (1983)†
Amino acids				
Killifish	Omnivore	Tryptophan	1.5*	Huang and Rout (1967)
Killifish	Omnivore	Cycloleucine (2 components)	0.03 and 0.12	Miller and Kinter (1979)†
Smooth dogfish	Carnivore	Cycloleucine	4*	Read (1967)†
Goldfish	Omnivore	Methionine	0.25	Kitchin and Morris (1971)†
		Valine	1.9	
Goldfish	Omnivore	Alanine	16.7*	Smith and Ellroy (1971)†
Trout	Carnivore	Leucine	2.7	Ingham and Arme (1977)†
		Methionine	1.9	
		Valine	2.4	
Trout	Carnivore	Glycine	52.5	Boge *et al.* (1979a)
Tench	Omnivore	Phenylalanine	2.9	Cartier *et al.* (1979)†
Surgeonfish, upper intestine	Herbivore	Alanine	0.1	Ferraris (1982)†
Eel, upper intestine	Carnivore	Alanine	1.5	Ferraris (1982)†
Eel, lower intestine	Carnivore	Alanine	0.2	Ferraris (1982)†

*Values in table calculated from data presented by authors.
†Studies which apparently fulfilled requirements for proper estimation of solute influx (see text).

Read (1967) was probably the first to correctly estimate the rates of transmembrane nutrient transport in fish intestines. He found a K_t^1 of 4 mM for cycloleucine, the uptake of which was inhibited by preincubating the tissue in galactose. Although some early influx studies used relatively long incubation intervals to estimate transmembrane nutrient movements (e.g. 30 min, Kitchin and Morris, 1971), more recent investigations have employed exposure intervals of considerably shorter duration (e.g. up to 3 min; Smith and Ellory, 1971; Miller and Kinter, 1979; Cartier *et al.*, 1979; Ferraris, 1982; Ferraris and Ahearn, 1983). In general, affinity constants (K_t^1) for brush border influx of sugars and amino acids in fish intestine range between 0.1 and 20 mM (Table 1), the only exception being the very high value reported for glycine influx in a rainbow trout midgut (Boge *et al.*, 1979a). In this instance the authors had not corrected their influx data for an apparent diffusion component (K_d^1) leading to a considerable underestimate of carrier affinity for this amino acid.

Transport constants and fish diet

In Table 1 a comparison of intestinal affinity constants of fishes with different diets, in studies which fulfilled the necessary requirements for accurately estimated solute influx (marked†), indicates that K_t^1 for amino acids were generally lower in herbivorous and omnivorous species (goldfish, killifish, surgeonfish) than in the carnivores (eel, trout, dogfish). This may be a result of possibly lower physiological amino acid concentrations in herbivorous intestines. The reverse situation occurs for the affinity constants of sugar influx, that is, the carnivorous species (eel) tend to have lower values for natural sugars than do the herbivores (surgeonfish) (Ferraris and Ahearn, 1983). Once again, this may reflect an adaptation to lower dietary sugar concentration in carnivorous fish intestines.

Comparison of K_t^1 and J_{max}^1 values of two different nutrients (one sugar and one amino acid) in the same fish also suggest that intestinal transport adaptations may occur in these animals in response to diet. Cartier *et al.* (1979) reported that K_t^1 and J_{max}^1 for phenylalanine in the omnivorous tench were almost 50% and 300% higher, respectively, than the kinetic constants for methylglucose (Table 1). The fish used in this study were raised on farms using commercial food pellets having a high protein content and, as a result, these kinetic constants may reflect responses to the artificial diets. In Ferraris (1982) and Ferraris and Ahearn (1983) influx kinetic constants for alanine and glucose were compared in upper and lower intestines of carnivorous and herbivorous fishes. These studies indicated that in eel (carnivore) upper intestine K_t^1 for alanine was five times higher than that for glucose, while in surgeonfish (herbivore) upper intestine the affinity constant for the sugar was six times that of the amino acid. In these latter two studies J_{max}^1 values were greatest where K_t^1 values were highest. Such results suggest that in fish upper intestine, transport mechanisms respond to diet, facilitating the transport of the more common nutrient via a greater number of carriers or a higher transport rate, while assuring the absorption of the less common nutrient through high affinity processes.

Fish lower intestine as a site of nutrient absorption

The affinity constants displayed in Table 1 generally summarize the findings of studies dealing with nutrient transport in fish upper intestine, that part of the gut analogous with the small intestine of mammals. While the adult mammalian lower intestine is incapable of active sugar transport (McNealy and Williams, 1929; Davidson and Garry, 1939; Long *et al.*, 1967) and active amino acid transport (Binder, 1970), there have been reports that fish lower intestines may be capable of facilitated or even active transport of these compounds (Musacchia, 1960; Mepham and Smith, 1966; Masacchia *et al.*, 1964; Smith, 1969; Sastry *et al.*, 1977a; Kitchin and Morris, 1971; Boge *et al.*, 1979a,b; Dabrowski and Dabrowska, 1981; Ferraris, 1982; Ferraris and Ahearn, 1983). Most of the fish reported to have hindgut carrier-mediated nutrient transport were either carnivorous or omnivorous in dietary preference and typically possessed relatively short gastrointestinal tracts. The reported occurrence of saturable processes for sugar and amino acid transport in the lower intestine of these animals may be a physiological compensation for gut length. This suggestion is strengthened by several studies comparing intestinal histochemistry of several teleosts with different feeding habits and diets. These investigations indicated that alkaline phosphatase, an enzyme believed to be involved in active transport (Lincheer *et al.*, 1971), is distributed in both upper and lower intestines of carnivorous and omnivorous fishes, but only in the upper intestine or herbivores (Srivastava, 1966; Chao, 1973; Stroband *et al.*, 1979). In addition, Sastry (1975) reported that the activity of this enzyme was higher in the upper intestine of the omnivore, *Heteropneustes fossilis*, than in that of the herbivore, *Cirrhinus reba*, and that there was a decline in alkaline phosphatase activity in both fishes toward the rectum.

Lower intestine nutrient transport affinity constants (K_t^1) from influx studies comparing carnivores and herbivores have generally been lower in both groups of fishes than those occurring in the upper intestines of the same fishes (Table 1). In all cases the J_{max}^1-values were two to six times lower in the hindguts (Boge *et al.*, 1979a; Ferraris, 1982). The implication of this relationship is that the fish lower intestine functions as a scavenger, removing the remaining nutrient that is more common in the respective diet.

Comparison of intestinal affinity constants between fish and other animals

Affinity constants for intestinal organic solute transport in mammals are generally higher than those of fishes. In rat and rabbit intestine the following K_t^1-values were reported: alanine (9.0 mM), glycine (23.0 mM), glucose (1.4, 8.0 mM), 3-*O*-methylglucose (18.0 mM) (Parsons and Prichard, 1966; Goldner *et al.*, 1969; Peterson *et al.*, 1970; Curran *et al.*, 1971). Cartier *et al.* (1979) used identical methods for different species and reported that K_t^1-values for phenylalanine in guinea pig and dog were twice that of the tench. Kitchin and Morris (1971) also showed goldfish transport constants to be lower than those of mammals and suggested that animals may have more

binding sites for some amino acids. Karasov and Diamond (1981) compared intestinal active transport of glucose in mammals, lizards and fish and found lowest uptake rates in the fish. These results suggest that: (1) a higher concentration of amino acids and sugars may be found in mammalian small intestine under normal conditions and may reflect a need by homeothermic animals to consume and absorb a greater amount of food and to support their high metabolic activity; and (2) the presence of increased absorptive surface area in mammalian intestine (Reifel and Travill, 1979) may be able to compensate for the occurrence of low affinity transport systems.

Intestinal transport constants for many invertebrates, on the other hand, are similar or even lower than those of fish, reflecting possible low solute concentrations in their guts and/or a low metabolic requirement. In arthropods, intestinal K_t^1-values for glycine (0.36 mM; Ahearn, 1974), lysine (0.018 mM; Brick and Ahearn 1978), and glucose (0.17 mM; Ahearn and Maginniss, 1977) were one to two orders of magnitude lower than those reported for the same solute in mammals. In these animals, however, the hepatopancreas may withdraw a substantial portion of the ingested food mass before it reaches the intestine (Ahearn *et al.*, 1983) so that the high affinity transport mechanisms found in the latter location may serve a scavenger role, as in fish lower intestine, absorbing what nutrients remain in the food bolus prior to defecation. In other invertebrates, not possessing an absorptive hepatopancreas, intestinal affinity constants were somewhat higher than those in crustaceans and similar to reported values of fishes. Bamford and Steward (1973) reported K_t^1-values for L-alanine and L-lysine of 0.63 mM and 0.43 mM, respectively, in the intestine of the polycheate worm *Arenicola marina* and a K_t^1 of 1.6 mM for glucose in everted intestinal sacs of the echinoderm *Thyone*. These studies across phyletic lines suggest that several factors may influence the affinity constants of intestinal transport processes for organic solutes. Among the most obvious factors are: (1) general organismic metabolic requirements; (2) dietary preferences; and (3) anatomical and physiological differentiation of absorptive regions of the gut.

Non-saturable nutrient influx

Several studies of nutrient influx across fish intestinal brush border membranes have disclosed a transfer mechanism for a variety of solutes that does not exhibit saturation even at high luminal substrate concentrations. None of these investigations unequivocally established this non-saturable transport component as being a carrier mechanism, which did not illustrate Michaelis-Menten kinetics within the substrate range used, or diffusion of the respective solute across the apical cell pole. However, this "apparent diffusion" of nutrient across fish intestinal brush border seems a widespread occurrence in these animals and solute transfer by the combination of at least one carrier mechanism plus non-saturable movement as described by equation (2) may be the rule in many lower vertebrates rather than the exception. Non-saturable transport components (K_d^1) for sugars and amino acids reported for fish intestines are presented in Table 2.

Table 2 indicates that non-saturable amino acid transport in freshwater fishes (goldfish and rainbow trout) appears to be less than that for marine fishes (surgeonfish and eel). Such a difference between fish groups might be related to the fact that euryhaline fish intestines were observed to undergo ultrastructural and electrophysiological changes (Oide and Utida, 1967; Utida *et al.*, 1967; Skadhauge, 1974; Ando, 1975) when moving from fresh water to seawater, exhibiting less transepithelial resistance in the process. Among the alterations observed were general increases in: (1) ionic permeability; (2) osmotic permeability to water; and (3) the molar ratio of water to sodium in the fluid moving across the intestinal wall. This increase in ionic permeability and passive water absorption may have been due, at least in part, to an increase in the number of aqueous pores in the epithelial brush border membrane, rendering it less hydophobic. Coincident with an increase in permeability to water and ions, the general permeability of this membrane for other small hydrophilic molecules such as sugars and amino acids may rise with seawater adaptation and explain the relatively high (K_d^1) values in these animals. The concept of non-saturable, Na^+-independent, simple diffusion-like absorption of water soluble organic molecules by the vertebrate intestine has been previously proposed for the entry of the vitamins *p*-aminobenzoic acid (Spencer *et al.*, 1966) and pyridoxine (Middleton, 1977; Hamm *et al.*, 1979; Mehansho *et al.*, 1979).

Smith and Kemp (1971) found that, in the goldfish, the time course of fatty acid composition change of

Table 2. Non-saturable influx constants of organic solutes in isolated fish intestines

Fish	Organic solute	K_d^1 (nmol cm^{-2} min^{-1} mM^{-1})	Reference
Sugars			
Killifish	Galactose	3.6	Huang and Rout (1967)
Eel, upper intestine	Glucose	3.9	Ferraris and Ahearn (1983)
Eel, lower intestine	Glucose	1.0	Ferraris and Ahearn (1983)
Surgeonfish, upper intestine	Glucose	3.8	Ferraris and Ahearn (1983)
Surgeonfish, lower intestine	Glucose	4.4	Ferraris and Ahearn (1983)
Amino acids			
Goldfish	Valine	1.7	Kitchin and Morris (1971)
Trout	Leucine	6.0	Ingham and Arme (1977)
Killifish	Tryptophan	9.7	Huang and Rout (1967)
Killifish	Cycloleucine	5.0	Miller and Kinter (1979)
Eel, upper intestine	Alanine	6.3	Ferraris (1982)
Eel, lower intestine	Alanine	1.5	Ferraris (1982)
Surgeonfish, upper intestine	Alanine	7.6	Ferraris (1982)
Surgeonfish, lower intestine	Alanine	10.0	Ferraris (1982)

intestinal membranes correlated well with changes in amino acid transport. If non-saturable transfer is dependent on membrane structure as reflected by fatty acid composition, then (K_d^1) values may be different between marine and freshwater fish intestines because of biochemical dissimilarities in membrane structure which in turn leads to differences in function. Indeed, freshwater fish, in general, seem to have higher levels of less polar fatty acids in their tissues than are found in those of seawater fishes (Ackman, 1967).

Effects of luminal Na^+ on nutrient influx

In mammalian intestine numerous investigations have shown that the absence of Na^+ in the luminal solution markedly alters the K_t^1 and/or J_{max}^1 of sugar and amino acid influx (Curran *et al.*, 1967; Schultz *et al.*, 1967; Goldner *et al.*, 1969). Only one similar detailed kinetic study has been done with fish intestines, using a euryhaline species whose luminal contents *in vivo* may range in Na^+ concentration from 10 to 500 mM, depending on the salinity of the environment (Smith and Ellory, 1971). These authors showed that the absence of Na^+ from the incubation medium increased the apparent affinity constant (K_t^1) of the carrier for alanine, but did not change the maximal rate of influx, a finding similar to that for alanine uptake in the rabbit ileum (Curran *et al.*, 1967). Kitchin and Morris (1971) and Miller and Kinter (1979) reported that absence of luminal Na^+ in influx experiments leaves only the non-mediated components of amino acid influx in goldfish and killifish intestines, suggesting that mediated components may be totally Na^+-dependent. Naftalin and Kleinzeller (1981) calculated the unidirectional fluxes of methylglocoside at the luminal cell surface of flounder intestinal epithelium and found much lower values than those for this carbohydrate in rabbit ileum. They suggested that reduced values in fish reflect a relative deficiency of Na^+-dependent sugar transport sites in the intestinal brush border of the lower vertebrates. Cartier *et al.* (1979) examined the effect of Na^+ deletion on 2 min influxes of phenylalanine and β-methyl-glucoside into tench intestine and disclosed 60–80% reductions in entry rates under these conditions compared to those of the control with this cation. Using brush border membrane vesicles of winter flounder intestine, Eveloff *et al.* (1980) showed that D-glucose and L-alanine uptake was stimulated by Na^+, while Boge and Rigel (1981) reported the same findings for D-glucose, aminoisobutyric acid, and glycine uptake in membrane vesicles from the herbivorous marine fish, *Boops salpa*. These studies indicate that while there is strong evidence for Na^+-dependent organic solute transport across intestinal brush border membranes in fish, comparative kinetic details of the interactions between ion, nutrient, and membrane transporter are clearly lacking in this group of vertebrates.

Nutrient structural requirements for carrier transport

Competitive and non-competitive interactions between sugars and amino acids at the intestinal brush border have been studied in detail in mammals and considerable information about substrate conformational requirements of a variety of carrier proteins has been forthcoming as a result of this work (Christensen, 1975). Several distinct amino acid carrier systems, each generally accepting only a limited number of substrates, occur in the mammal small intestine. Similar, although not as rigidly defined, properties described carbohydrate transport systems. Even though distinct carrier types are present in mammal gut, some overlap of transported substrates may occur between them. In rabbit ileum or rat small intestine for example, several neutral amino acids can exert a variety of transport effects on other substrates as a result of their cross-reactivity at several carrier loci. Thus, these neutral amino acids may enter the absorptive cell by two distinct saturable carrier mechanisms, they are also capable of using the carrier for cationic amino acids, and they are able to transstimulate the uptake of some basic amino acids and inhibit the absorption of sugars and other neutral amino acids (Newey and Smyth, 1964; Alvarado, 1966; Chez *et al.*, 1966; Schultz and Curran, 1970; Reiser and Christiansen, 1971; Sepulveda and Smith, 1978; Paterson *et al.*, 1979; Munck, 1981).

There have been a limited number of studies defining carrier specificity for nutrients in fish intestines. Miller and Kinter (1979) showed that influx of cycloleucine into killifish intestine occurred by three pathways: (a) one shared with all neutral amino acids; (b) one shared with neutral D- and L-amino carboxylic acids, but not with β-alanine or taurine; (c) one that was non-mediated. On the other hand, Ingham and Arme (1977) stated that while L-leucine, L-methionine and L-valine were mutually competitive inhibitors of one influx system in rainbow trout intestine, these authors also found that this uptake system exhibited stereospecificity and did not accept the D-form of these amino acids. Sugar–amino acid interactions were suggested by Read (1967) when he showed that cycloleucine uptake in the dogfish intestine was competitively inhibited by galactose, glucose, 3-*O*-methyl-D-glucose and α-methylglucoside. Earlier, Read *et al.* (1960) had found that there was competitive inhibition of one amino acid by another in this tissue. Cartier *et al.* (1979) reported that β-methylglucoside and galactose inhibit each other's influx during 2 min incubations in the tench, presumably through interactions at a common transport locus. Finally, Naftalin and Kleinzeller (1981) discovered an Na^+-dependent, galactose-inhibited secretory system in flounder intestine that could actively transport 2-deoxy-D-galactose from epithelium to mucosal bathing solution.

As these studies suggest, nutrient carrier mechanisms in fish gut display some substrate structural specificities, but the extent of these requirements, and their possible relationship to mammalian carrier properties, remain to be explored.

STEADY-STATE NUTRIENT ACCUMULATION BY FISH INTESTINE

Nutrient T/M ratios in mammals and fishes

In higher vertebrates net transmural nutrient transport occurs as a two-step process (Schultz and Zalusky, 1964a,b; Schultz, 1977). First, amino acids and sugars are cotransported across the brush border

Table 3. Steady-state sugar amino acid accumulation by isolated fish intestines

Fish	Organic solute	Saline concentration (mM)	Estimated intracellular concentration (mM)	T/M ratio*	Reference
Sugars					
Freshwater fish					
Trout	Glucose	5.2	11.6	2.2	Stokes and Fromm (1964)
Tench	β-Methyl-glucoside	1	7	7	Cartier *et al.* (1979)
Toadfish	Glucose	1	5.8	5.8	Farmanfarmaian *et al.* (1972)
Seawater fish					
Winter flounder	Galactose	1	1	1	Naftalin and Kleinzeller (1981)
Winter flounder	2-Deoxy-galactose	1	2.4	2.4	Naftalin and Kleinzeller (1981)
Surgeonfish, upper intestine	Glucose	0.05	0.04	0.8†	Ferraris (1982)
Surgeonfish, lower intestine	Glucose	0.05	0.04	0.8†	Ferraris (1982)
Eel, upper intestine	Glucose	0.05	0.03	0.6	Ferraris (1982)
Eel, lower intestine	Glucose	0.05	0.06	1.2†	Ferraris (1982)
Amino acids					
Freshwater fish					
Trout	Leucine	5.0	10.35	2.1	Ingham and Arme (1977)
Tench	Phenylalanine	1.0	5.0	5.0	Cartier *et al.* (1979)
Killifish	Cycloleucine	0.01	0.04	4.0	Miller and Kinter (1979)
Goldfish, anterior intestine	Methionine	0.02	0.066	3.3	Kitchin and Morris
Goldfish, posterior intestine	Methionine	0.02	0.068	3.4	Kitchin and Morris (1971)
Goldfish, anterior intestine	Valine	0.02	0.08	4.0	Kitchin and Morris (1971)
Goldfish, posterior intestine	Valine	0.02	0.066	3.3	Kitchin and Morris (1971)
Seawater fish					
Surgeonfish, upper intestine	Alanine	0.05	0.03‡	0.6	Ferraris (1982)
Surgeonfish, lower intestine	Alanine	0.05	0.02‡	0.4	Ferraris (1982)
Eel, upper intestine	Alanine	0.05	0.01‡	0.2	Ferraris (1982)
Eel, lower intestine	Alanine	0.05	0.01‡	0.2	Ferraris (1982)

*T/M (tissue/medium) ratio is calculated by dividing the estimated intracellular concentration by the saline concentration of the respective organic solute.
†Values not significantly different ($P < 0.05$) from a T/M ratio of 1.0.
‡Low intracellular organic solute concentration due to extensive metabolic transformation of labelled alanine.

membrane with sodium ions using the energy inherent in the transapical Na^+-gradient to accumulate the organic solutes to intracellular concentrations that exceed those of either the lumen or the blood. Second, the intracellular nutrients pass across the basolateral membranes by facilitated diffusion down their concentration gradients to the blood. For this two-step process to work efficiently, the brush border membrane must have a relatively low permeability to the organic solutes so that they do not diffuse from the cell to the lumen, and intracellular metabolism of the nutrients must be minimal to maintain an effective diffusion gradient for these molecules across the basolateral cell pole. As a result of this two-step process, active transport of sugars and amino acids by intestine of higher vertebrates is often characterized by a T/M (tissue/medium) concentration ratio, which is the extent to which organic molecules can be accumulated intracellularly to concentrations in excess of those in the lumen. The T/M ratio for intestinal sugar accumulation is 3.6 in the hamster (Bihler and Crane, 1962), between 3.5 and 5.5 for rabbit (Goldner *et al.*, 1969; Holman and Naftalin, 1976), and around 4.0 in frog (Armstrong *et al.*, 1973). Amino acid accumulation by higher vertebrate intestine is often greater than that reported for sugars. In rat jejunum the T/M ratios for lysine and leucine are 9.7 and 7.0, respectively (Munck, 1981).

Table 3 presents a series of steady-state T/M ratios for sugars and amino acids in the intestines of freshwater and marine fishes. With the exception of the non-metabolizable analog sugars (2-deoxygalactose; Naftalin and Kleinzeller, 1981) and amino acids (cycloleucine; Miller and Kinter, 1979), a clear pattern occurs among the fishes with regard to intestinal accumulation of natural organic solutes. While freshwater fishes, without exception, have intestinal T/M ratios for both sugars and amino acids exceeding unity, indicative of accumulation from the lumen against a concentration gradient, marine fish gut is incapable of achieving T/M ratios for these molecules greater than 1.0 (Ferraris, 1982).

The inability of marine fish gut to inhibit a T/M ratio exceeding unity for natural organic solutes may be due to the combination of a relatively permeable brush border membrane in these animals compared to that displayed by freshwater species and by higher vertebrates (Table 2), and the occurrence of substantial nutrient metabolism by epithelial cells following transfer across the apical membrane from the lumen (Stokes and Fromm, 1964; Mepham and Smith, 1966; Ferraris, 1982). During nutrient uptake a greater apparent diffusion process (K_d') for organic solutes in the brush border of marine fish intestine than in that of other vertebrates would facilitate the back diffusion of unmetabolized nutrients to the lumen as the intracellular concentration began to increase. At steady-state, Na^+-dependent influx of nutrient from lumen to cell could closely match the combination of non-saturable nutrient efflux from cell to lumen plus a small organic solute transfer across the basolateral membrane to the blood. Indeed, backflux or secretion of nutrients from epithelial cells of the luminal solution seems a common occurrence in fishes (Wilson, 1957; Stokes and Fromm, 1964; Musacchia *et al.*, 1966; Huang and Rout, 1967; Neff and Musacchia, 1967; Smith, 1969; Boge *et al.*, 1981; Dabrowski and Dabrowska, 1981; Naftalin and Kleinzeller, 1981). Intracellular metabolism of a transported nutrient would reduce both backflux of the molecule to the lumen as well as transfer to the blood. Efflux of metabolites from epithelial cell to blood across the basolateral cell border may also play an important, but as yet undisclosed role, in marine fish nutrition. In general, information concerning the nature of basolateral nutrient transport in these animals is currently lacking. A knowledge of basolateral carrier affinities, the possible coupling of organic solute flow across this membrane with ions or with cellular energy supplies, and the determination of membrane nutrient and metabolite permeabilities in this location are needed to clarify epithelial absorptive processes in marine fishes.

Effects of luminal Na^+ *on steady-state nutrient accumulation*

Absence of luminal Na^+ markedly depresses the steady-state uptake and/or T/M ratios of nutrient transport in some freshwater teleost and elasmobranch intestines. Read (1967) showed that galactose and cycloleucine transport in the dogfish were significantly reduced when the Na^+ concentration was decreased from 225 to 50 mM. Ingham and Arme (1977) reported that T/M ratios of leucine absorption in rainbow trout decreased from 1.9 to 1.3 with increasing replacement of Na^+ by Tris, choline or lithium. In the tench, Cartier *et al.* (1979) found that uptake of phenylalanine or β-methylglucoside was reduced by 80 to 95% from control values when Na^+ was substituted with K^+ or choline. None of these studies specifically identified the role of Na^+ in steady-state nutrient accumulation by fish intestine, although cation modification of sugar and amino acid influxes across the epithelial brush border most likely contributed to observed patterns. Intracellular effects of Na^+ on epithelial nutrient metabolism or influences of this ion on basolateral transport events during steady-state remain unstudied.

Inhibition of steady-state concentrative nutrient uptake

Isolated freshwater fish intestines which exhibit steady-state nutrient T/M ratios greater than unity (Table 3) and, therefore, normally demonstrate active transport of sugars and amino acids under control conditions may have this ability impaired in the presence of metabolic inhibitors and/or other nutrients. For example, Ingham and Arme (1977) reported a 16.5–45.2% reduction of T/M ratio of L-leucine absorption in the presence of cyanide, iodoacetate and dinitrophenol, and a 23–59% decrease in the presence of other neutral amino acids. After 2 hr of incubation, Hokazono *et al.* (1979) found that L-lysine transport in rainbow trout intestine was stimulated by ATP, but inhibited by D-glucose. Ouabain, harmaline and dinitrophenol also reduced steady-state uptake of phenylalanine and β-methylglucoside by about 50–85% in the tench (Cartier *et al.*, 1979). On the other hand, sodium fluoride and dinitrophenol only slightly inhibited concentrative uptake of glycine in rainbow trout intestine, whereas anoxia and ouabain were much more effective (Boge *et al.*, 1979b).

Possible inhibitory effects of metabolic poisons or competing substrate molecules on steady-state uptake or T/M ratios of sugars and amino acids by marine fish intestines have not been examined. In light of the inability of seawater fish gut to exhibit T/M ratios exceeding unity in the presence of luminal Na^+ (Table 3), careful selection of inhibitors with known intracellular or plasma membrane effects could be helpful in describing the mechanisms of transmural nutrient transport in these animals.

TRANSEPITHELIAL FLUXES

Studies reporting transepithelial fluxes of nutrients across isolated fish intestines, prepared as tissue sheets in Ussing-type chambers or as everted sacs, either measured unidirectional mucosa to serosa (J^i_{ms}) and serosa to mucosa (J^i_{sm}) fluxes of the test solute across the preparation and established the net flux (J^i_{net}) by subtraction, or determined the net solute movement directly through chemical analysis of the solutions bathing the two tissue surfaces. These experiments were usually conducted in the absence of both solute concentration gradients and transmural electrical potential differences across the tissue so that a net flux would strongly suggest the occurrence of an active absorption or secretion mechanism in the epithelium. Values of net transepithelial sugar and amino acid transport in upper and lower intestines of a variety of fish species are presented in Table 4. In addition, estimated net fluxes of the respective substances at a fixed concentration (0.05 mM) are also shown in this table, assuming a linear relationship between luminal solute concentration and net solute flux.

Transepithelial sugar fluxes in fish upper intestine

Table 4 indicates that, in general, freshwater teleost upper intestine (catfish, rainbow trout) displays greater net transepithelial sugar fluxes than those occurring across the upper intestine of marine species when calculated at a common luminal solute concentration. Part of this significant difference between fish groups may be a result of structural and permeability dissimilarities in the epithelial brush border membrane discussed above. As suggested in Table 2, seawater-adapted teleost intestine appears to exhibit a higher apparent diffusion constant (K^i_d) toward organic solutes than does that of the freshwater animals. While this transport component may facilitate the downhill flow of nutrients from lumen to cell during conditions of low intracellular glucose concentration, it would also lead to the loss of accumulated intracellular sugars across the mucosal membrane if luminal glucose concentrations were low. Therefore, marine teleost intestine, possessing a large apparent diffusion process for organic solutes in the brush border membrane, exhibits a relatively low transepithelial nutrient transfer as a result of significant backflux of organic solutes to the gut lumen from the epithelial cells. In freshwater species, the relatively low apical membrane permeability reduces this nutrient backflux, facilitating net solute flow to the blood from the gut lumen.

Like influxes and steady-state uptake, transepithelial transport of sugars in fishes has been shown to be inhibited by the same factors that cause reduced sugar transport in higher vertebrates. These factors include a decrease in temperature, absence of luminal Na^+, anoxia, phlorizin, phloretin, ouabain, sodium azide, iodoacetic acid, cyanide, uranyl acetate and dinitrophenol (Musacchia, 1960; Carlisky and Huang, 1962; Musacchia *et al.*, 1964, 1966; Huang and Rout, 1967; Chen and Huang, 1972; Escoubet *et al.*, 1973; Sastry *et al.*, 1977a,b). Similar conditions or compounds generally have the same effect on transepithelial amino acid transport in fish gut (Rout *et al.*, 1965; Neff and Musacchia, 1967; Huang and Rout, 1967; Smith, 1969; Chen and Huang, 1972).

Quantitatively, net sugar fluxes across fish upper intestine are considerably slower than those reported for other vertebrates. Huang and Chen (1975) reported 100% faster intestinal transepithelial glucose fluxes in hamster than in catfish. Calculated transepithelial intestinal glucose and 6-deoxyglucose fluxes at a luminal concentration of 0.05 mM in turtle and squirrel are 4000 and 4400 pmol cm^{-2} min^{-1} at 30°C (Fox, 1961; Crane and Wilson, 1958), suggesting that absorption of sugars in fish might be as much as an order of magnitude lower than that of the higher vertebrates. Much of this difference may lie in metabolic dissimilarities between the vertebrate groups.

Transepithelial amino acid fluxes in fish upper intestine

Net transepithelial fluxes of amino acids across teleost upper intestine displayed in Table 4 indicate that the absorption of these molecules occurs considerably faster than does that for monosaccharides. Cheng and Huang (1972) showed that, in catfish, net tyrosine fluxes across the intestine were about 3-fold those of glucose and galactose. In killifish even larger differences between the two nutrient groups were disclosed; tyrosine and tryptophan absorption was approximately ten-fold that of glucose and galactose (Huang and Rout, 1967). Finally, in tench, Cartier *et al.* (1979) showed a 6-fold greater net phenylalanine movement to the blood than that for β-methylglucoside. Fish appear to exhibit a preference for protein as a nutrition source when they are cultivated (Cowey and Sargent, 1979; Brett and Groves (1979), and in many cases fish intestinal epithelial cells can accumulate amino acids to higher concentrations than those of sugars. Greater luminal amino acid concentrations from protein hydrolysis combined with higher intracellular concentration ratios may be, at least partially, responsible for the large net flow of amino acids from lumen to blood in these animals.

Transepithelial nutrient fluxes in fish lower intestine

Very little is known about comparative nutrient fluxes across the vertebrate lower intestine. The only studies on sugar and amino acid transport in this part of the fish gastrointestinal tract are those shown in Table 4 by Musacchia *et al.* (1966), Neff and Musacchia (1967), Ferraris (1982) and Ferraris and Ahearn (1983). In most cases a significant net flux of the respective nutrient was observed in lower intestine, suggesting that this part of the gut in fishes may play a role in total absorption. Indeed, net transepithelial sugar and amino acid fluxes were larger in the lower intestines of the carnivores (eel, scup, puffer)

Table 4. Net transepithelial fluxes of organic solutes across isolated fish intestines

Fish	Organic solute	Concentration of solute (mM)	Transepithelial flux (pmol $cm^{-2} min^{-1}$)	Estimated Flux at 0.05 mM (pmol $cm^{-2} min^{-1}$)	Reference
Sugars					
Catfish	Glucose	0.25	6933	1386	Musacchia *et al.* (1964)
Catfish	Glucose	5	26,000	260	Chen and Huang (1972)
Catfish	Galactose	5	20,160	202	Chen and Huang (1972)
Killifish	Glucose	11	8333	38	Huang and Rout (1967)
Killifish	Galactose	11	17,973	82	Huang and Rout (1967)
Dogfish	Glucose	12	370,000	1541	Carlisky and Huang (1962)
Winter flounder	Galactose	1	217	11	Naftalin and Kleinzeller (1981)
Winter flounder	2-Deoxy-galactose	1	2067	103	Naftalin and Kleinzeller (1981)
Tench	β-Methyl-glucoside	1	683	34	Cartier *et al.* (1979)
Scup, upper intestine	Glucose	0.5	1066	107	Masacchia *et al.* (1966)
Scup, lower intestine	Glucose	0.5	2068	207	Musacchia *et al.* (1966)
Puffer, upper intestine	Glucose	0.5	867	87	Musacchia *et al.* (1966)
Puffer, lower intestine	Glucose	0.5	1200	210	Musacchia *et al.* (1966)
Trout	Glucose	2.5	16,000	320	Stokes and Fromm (1964)
Surgeonfish, upper intestine	Glucose	0.05	21	21	Ferraris and Ahearn (1983)
Surgeonfish, lower intestine	Glucose	0.05	4	4	Ferraris and Ahearn (1983)
Eel, upper intestine	Glucose	0.05	59	59	Ferraris and Ahearn (1983)
Eel, lower intestine	Glucose	0.05	284	284	Ferraris and Ahearn (1983)
Amino acids					
Catfish	Tyrosine	1	14,000	700	Chen and Huang (1972)
Catfish	Leucine	10	18,666	93	Neff and Musacchia (1967)
Tench	Phenylalanine	1	3416	171	Cartier *et al.* (1979)
Winter flounder	Tyrosine	2.5	8000	160	Rout *et al.* (1965)
Winter flounder	Tryptophan	2.0	4678	117	Rout *et al.* (1965)
Scup, upper intestine	Leucine	10	18,666	93	Neff and Musacchia (1967)
Scup, lower intestine	Leucine	10	23,330	117	Neff and Musacchai (1967)
Killifish	Tryptophan	2	16,600	415	Huang and Rout (1967)
Killifish	Tyrosine	2	20,000	500	Huang and Rout (1967)
White grunt	Glycine	3	59,000	983	Smith (1969)
Surgeonfish, upper intestine	Alanine	0.05	42	42	Ferraris (1982)
Surgeonfish, lower intestine	Alanine	0.05	21	21	Ferraris (1982)
Eel, upper intestine	Alanine	0.05	18	18	Ferraris (1982)
Eel, lower intestine	Alanine	0.05	67	67	Ferraris (1982)

than in each of their upper intestines. Only in herbivorous fish upper intestine were larger net fluxes of nutrients observed than those exhibited by their lower intestine (Ferraris, 1982; Ferraris and Ahearn, 1983). Table 2 indicates that the apparent diffusion constant (K_d') value for glucose influx in eel lower intestine (1.0 nmol cm^{-2} min^{-1} mM^{-1}) was four times lower than those of the upper intestine of this fish or either part of the surgeonfish gut (Ferraris, 1982; Ferraris and Ahearn, 1983). As mentioned previously, a low apparent diffusion constant for the epithelial brush border membrane results in reduced solute backflux to the lumen and potentially increases the movement of a given solute to the blood. It is most likely because of this reduced K_d'-value in eel lower intestine that such large glucose and alanine net fluxes were observed. Similar arguments could be made for large net glucose/leucine fluxes in the lower intestines of scup and puffer (Table 4), but paucity of permeability data for these species prevents a firm conclusion on this issue.

ELECTROPHYSIOLOGY

Comparative aspects of intestinal electrophysiology

The electrical character of fish intestine has been examined in the presence and absence of luminal nutrients and results suggest considerable functional differences between these animals and the higher vertebrates. The intestines from mammals, birds, reptiles and amphibians generally have an electrically positive serosa, relative to the mucosa, which becomes more positive when actively transported organic solutes are added to the lumen (Table 5; Huang and Chen, 1975; Schultz, 1977). In contrast, fish intestines display a considerable electrical diversity (Table 5). Some species exhibit a serosal positive transmural potential difference (Mepham and Smith, 1966; Datta and Savage, 1968; Buclon, 1974; Ando *et al.*, 1975; Albus and van Heukelem, 1976), while others possess an electrically negative serosa (Huang and Chen, 1971; Chen and Huang, 1972; Huang and Chen, 1975; Ando *et al.*, 1975; Field *et al.*, 1978). There have also been some reports suggesting the absence of a transmural potential difference across fish gut (House and Green, 1963; Ando *et al.*, 1975). As shown in Table 5, it is mostly the freshwater fish species that demonstrate a serosa positive polarity, while marine teleosts, almost universally, have a negative serosa. Several studies have reported transmural potential difference values for the same fish species adapted to either fresh water to seawater (Ando, 1974; Ando *et al.*, 1975; Ando and Kobayashi, 1978; Smith *et al.*, 1975). In every fish species examined in this manner, the transmural potential difference became more serosally negative when freshwater individuals were adapted to seawater.

In higher vertebrates and some invertebrates the serosal positive transmural potential difference is the result of a strong net flux of sodium from lumen to blood (Rose and Schultz, 1971; Schultz, 1977; Quay and Armstrong, 1969; Ahearn, 1980). The same may also apply to freshwater fishes exhibiting a serosa positive electrical polarity, although a clear demonstration of this has yet to be shown. The serosal negativity of marine fish intestine is unique among the vertebrates and has been more difficult to explain. Two models have been proposed to clarify the nature of epithelial activities leading to an electrically negative intestinal serosa. Huang and Chen (1971) reported that the Na^+-independent net Cl^- flux from lumen to blood in winter flounder was greater than the net Na^+ flux in the same direction. Later, Ando *et al.* (1975) observed that the net transmural fluxes of Na^+ and Cl^- were equal when euryhaline fishes were in fresh water, but in seawater the serosally-directed Cl^- flux alone increased in the short-circulated intestine. These latter authors postulated the development of an electrogenic chloride pump in the intestines of seawater-adapted fishes to explain the increase in net transmural chloride flux. They did not define the anatomical location of such a pump or elaborate upon any of its biochemical properties. More recently, Field *et al.* (1978) proposed a model to explain the serosal negativity of flounder gut which did not invoke an electrogenic chloride pump, but rather suggested that differences in Na^+ and Cl^- permeabilities and mobilities in epithelial tight junctions and lateral spaces would create diffusion potentials across the epithelium of the observed polarity.

Table 5 indicates that fish intestinal short-circuit currents (SCC) are quantitatively similar to those displayed by guts of other vertebrate groups implying similar capabilities to affect a net ion movement across the tissues, although the direction of this net ion flow and the ion species of which it is composed may vary. Table 5 also summarizes the transmural electrical resistances for several species of freshwater and marine fishes as well as those for guts from other vertebrate groups. In general, vertebrate intestine is a low resistance epithelium, often several hundred ohms cm^{-2} lower than high resistance tissues such as amphibian skin or bladder. This low electrical resistance in vertebrate gut is generally due to paracellular and junctional complexes that offer little resistance to ion flow, reducing the magnitude of the transepithelial electrical potential difference that can be generated. Transepithelial resistances of marine fish intestine are among the lowest values recorded (Ando *et al.*, 1975; Field *et al.*, 1978; Ferraris and Ahearn, 1983). In euryhaline fishes, intestinal resistance appears to decrease significantly when fresh water-adapted individuals are placed in seawater (compare rainbow trout and Japanese eel values in Table 5; Ando *et al.*, 1975). These results suggest that there may be a functional link between changes in epithelial resistance observed in teleosts undergoing salinity adaptation and the simultaneous reversal in transmural potential difference that is often recorded in the same species.

Effects of organic solutes on transmural potential difference

In amphibians and mammals, luminal addition of sugars or amino acids results in a rapid depolarization of the brush border membrane electrical potential difference, making the cytoplasm less negative, relative to the mucosa, due to electrogenic cotransport of the organic solute and sodium ion across this cell pole (Rose and Schultz, 1971; White and Armstrong, 1971). This event is followed by a hyperpolarization of the transepithelial potential

Table 5. Comparative electrical properties of vertebrate intestines

Animal	Transmural potential difference (mV)	Short-current current (μamp cm^{-2})	Resistance (ohm cm^{-2})	Reference
Fish				
Freshwater				
Sculpin	0	0	—	House and Green (1963)
Goldfish	2	—	—	Mephan and Smith (1966)
Goldfish	4	—	—	Smith (1966)
Goldfish	0.1	1	106	Albus and van Heukelem (1976)
Catfish	2.5	—	—	Datta and Savage (1968)
Catfish	−1.7	−10	170	Chen and Huang (1972)
Tench	1.5	14	107	Buclon (1974)
Trout	0.2	2	108	Ando *et al.* (1975)
Carp	1.4	30	48	Ando *et al.* (1975)
Parasilurus asotos	1.1	25	44	Ando *et al.* (1975)
Channa argus	0.6	7	80	Ando *et al.* (1975)
European flounder	−1.2	−18	67	Smith *et al.* (1975)
Anguilla eel, anterior intestine	−0.5	−4.2	88	Ando *et al.* (1975)
Anguilla eel, posterior intestine	0.1	1.0	76	Ando *et al.* (1975)
Seawater				
European flounder	−1.9	−45	42	Smith *et al.*(1975)
Anguilla eel, anterior intestine	−2.5	−42	69	Ando *et al.* (1975)
Anguilla eel, posterior intestine	−2.6	−57	53	Ando *et al.* (1975)
White flounder	−2.9	−24	121	Huang and Chen (1971)
White flounder	−3–−7	−60–−140	50	Field *et al.* (1978)
Eel	−2.8	−18	156	Huang and Chen (1971)
Trout	−0.9	−25	43	Ando *et al.* (1975)
Prionorus microlepidotus	0.06	0.4	112	Ando *et al.* (1975)
Gonistius zonatus	−2.9	−76	48	Ando *et al.* (1975)
Astroconger myriaster	−3.2	−50	65	Ando *et al.* (1975)
Grey mullet	0.0	—	—	Ando *et al.* (1975)
Moray eel, upper intestine	−1.6	−18	88	Ferraris and Ahearn (1983)
Moray eel, lower intestine	−2.2	−37	59	Ferraris and Ahearn (1983)
Surgeonfish, upper intestine	−0.1	−2.5	40	Ferraris and Ahearn (1983)
Surgeonfish, lower intestine	−0.6	−14	42	Ferraris and Ahearn (1983)
Mammals				
Rat	0.8	28	29	Barry *et al.* (1965)
Rat	2.6	54	48	Lyon (1967)
Rabbit	5.4	90	60	Schultz and Zalusky (1964a,b)
Dog	0.3	4	86	Huang and Chen (1975)
Hamster	2.3	30	77	Huang and Chen (1975)
Monkey	2.6	22	118	Huang and Chen (1975)
Birds				
Chicken	1.8	12	146	Huang and Chen (1975)
Reptiles				
Turtle	5.1	14	351	Huang and Chen (1975)
Amphibians				
Bullfrog	1.2	8	150	Quay and Armstrong (1969)
Frog	1.5	12	125	Huang and Chen (1975)

difference, making the serosa more positive, relative to the mucosa, as the cotransported sodium ion is transferred across the basolateral membrane by an electrogenic sodium pump. The overall effect of adding nutrients to the mucosal surface of higher vertebrates, therefore, is the elevation of transmural potential difference, short-circuit current, and net sodium flux (Schultz and Zalusky, 1964a,b; Barry *et al.*, 1964; Smith, 1966; Rose and Schultz, 1971; White and Armstrong, 1971).

Addition of either sugars or amino acids to the intestinal lumen of freshwater fishes such as the goldfish or tench results in a rapid and marked (1.7–2.5 mV change) hyperpolarization of the spontaneous serosa positive transmural potential difference (Smith, 1966; Albus and van Heukelem, 1976; Buclon, 1974). Glucose added to the intestinal lumen of marine fishes, on the other hand, leads to a small change in the spontaneous transmural potential difference of a more prolonged duration (Ferraris and Ahearn, 1983). However, in these seawater fishes the glucose-evoked change in transmural potential difference is of the same polarity as that obeserved in freshwater species, i.e. a serosa negative spontaneous potential difference becomes less negative with glucose. The relationship between luminal glucose concentration and change in spontaneous transmural potential difference in these tissues is hyperbolic and follows the equation:

$$\Delta PD = \frac{\Delta PD^{G}_{max}[\mathrm{G}]}{K^{G}_{t} + [\mathrm{G}]} \tag{3}$$

where ΔPD is glucose-evoked change in transmural potential difference, ΔPD^{G}_{max} is the maximal glucose-evoked change in PD, K^{G}_{t} is the glucose concentration resulting in 50% ΔPD^{G}_{max}, and [G] is the luminal glucose concentration. This relationship suggests that the glucose-evoked change in PD is rate-limited by a process that illustrates saturation kinetics such as the brush border carrier mechanism described earlier for the influx of sugar into the epithelium from the luminal solution (Table 1). These results further imply that sugars or amino acids added to the mucosal surface of fish intestine may lead to the net flow of positively charged ions from lumen to blood as occurs in the guts of higher vertebrates.

Effects of organic solutes on transmural sodium flux and short-circut current

In both carnivorous (eel) and herbivorous (surgeonfish) marine fishes, the upper intestine actively transports sodium from lumen to blood (Ferraris and Ahearn, 1983). However, because the absorption of sodium in these animals is linked to the simultaneous absorption of chloride and the secretion of potassium (Musch *et al.*, 1982), the short-circuit current (SCC) measured across the tissue has a strong serosal negative polarity. Addition of glucose to the mucosal surface of either the eel or surgeonfish intestine leads to a significant reduction in the serosal negativity of the SCC and a small, but not quite significant, elevation in net transmural sodium transport from lumen to blood (Ferraris and Ahearn, 1983). These data, and those presented earlier in this review, suggest that at least in marine teleost intestine, cotransport of Na^+ and glucose across the epithelial brush border membrane in a manner similar to that described for higher vertebrates most likely occurs. This cotransport is followed by the extrusion of Na^+ from the cells across the basolateral membrane to the blood. The occurrence of linked transmural fluxes of Na^+, Cl^- and K^+ by independent mechanisms reduces the depolarizing influence that Na^+-glucose cotransport would otherwise have on this tissue and lowers the contribution of glucose-dependent Na^+ flux to the net transmural movement of this cation.

SUMMARY

The length, morphology and histology of fish intestines appear strongly influenced by feeding habits and diet. Those species with small relative gut lengths (gut length/total body length) increase absorptive capabilities by amplifying mucosal folding. In addition, species with short intestines such as carnivores exhibit significant nutrient absorption in their lower intestines, whereas those fish with longer guts (e.g. herbivores) have reduced amino acid and sugar transport in this location.

Influx studies indicate that amino acid and sugar transport across fish intestine brush border membranes from the luminal solution occurs by two or more mechanisms, at least one of these being a carrier-mediated Na^+-dependent system. The kinetic constant values (K_t^1 and J_{max}^1) of these saturable systems tend to reflect adaptations to diet and to expected luminal concentrations of each dietary component. A non-saturable nutrient influx pathway also occurs in fish epithelial brush border membranes and may be responsible for a considerable fraction of total uptake across this pole of the cell. This latter process may also be responsible for a significant nutrient backflux from epithelial cell to mucosal solution as intracellular concentrations rise, reducing the concentrative ability of the epithelium and lowering net transepithelial transport to the blood.

Freshwater fish, like higher vertebrates, can accumulate sugars and amino acids in the intestine to concentrations above those in the lumen; marine fish cannot. The failure of seawater species to transport nutrients against a concentration gradient within intestinal tissues is due to: (1) a large backflux or secretion of organic solutes from epithelium to lumen; and (2) significant intracellular metabolism of the molecules following influx. As a result of this combination, upper intestine of marine fishes exhibits a smaller nutrient absorption from lumen to blood than that displayed by freshwater species. Nutrient absorption in the lower intestine of seawater fishes may, to some extent, compensate for low rates of transepithelial transport in the upper gut.

Fish intestines display a considerable electrical diversity, with most fresh water species possessing an electrically positive serosa, while marine species demonstrate a negative serosa. Two models have been suggested for the negative serosa in marine animals: (1) the occurrence of an electrogenic Cl^- pump; or (2) the presence of intracellular spaces and cell junctions with differential ion permeabilities to Na^+ and Cl^- leading to diffusion potentials of the observed polarity. Addition of organic solutes to isolated freshwater fish intestines results in rapid and marked hyperpolarization of the positive serosa potential. In marine fishes, mucosal addition of nutrients generates only a small and slow depolarization of the serosa negative potential, probably because the low transepithelial resistance of marine fish intestines compromises any change in potential, and/or because linked transmural fluxes of Na^+, Cl^- and K^+ reduce the depolarizing influence of a Na^+-organic solute cotransport system.

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ALLOMETRIC RELATIONSHIP BETWEEN TISSUE RESPIRATION AND BODY MASS IN THE CARP

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Abstract—1. Respiration rate *in vitro* (Q_{O_2}) of most tissues in carp decreased with increasing body mass, with slopes less steep than that for the decline in weight-specific metabolic rate of intact carp.

2. The Q_{O_2} value of the whole body which was prepared in the same way as in tissues decreased, on the other hand, with a slope very close to the slope for intact carp.

INTRODUCTION

The relationship between basal metabolic rate (M) and body mass (W) is described by an allometric equation, $M = aW^b$. When the exponent b is smaller than unity, the metabolic rate per unit weight or weight-specific metabolic rate (M/W) decreases with increasing body mass as expressed by another equation, $M/W = aW^{b-1}$, where $(b-1)$ is a negative. This phenomenon or the decline in weight-specific metabolic rate with increasing body mass has been widely observed and repeatedly discussed interspecifically and intraspecifically with various animals both of homeotherms and poikilotherms (Brody, 1945; Bertalanffy and Pirozynski, 1953; Zeuthen, 1953; Winberg, 1956; Hemmingsen, 1960; Kleiber, 1961; Kayser and Heusner, 1964; Glass, 1969; Schmidt-Nielsen, 1970; Ricker, 1973; Brett and Groves, 1979; Heusner, 1982; and many others).

Several hypotheses have been proposed to interpret the phenomenon. One of them is an explanation based upon the decrease in the rate of tissue respiration per unit weight, Q_{O_2}, with increasing body mass. The value of Q_{O_2} has been examined with various tissues of various animals, but the results were not consistent. The values of Q_{O_2} were reported, in some cases, to decrease with similar slopes to those of metabolic rate of intact animals, with increasing body mass (Kayser *et al.*, 1925; Le Breton and Kayser, 1926; Weymouth *et al.*, 1942, 1944; Crandall and Smith, 1952; Bertalanffy and Pirozynski, 1953; Vilenkina, 1968). On the other hand, in some other cases, no decline or even a slight increase in Q_{O_2} was observed (Terroine and Roche, 1925; Crandall and Smith, 1952; Bertalanffy and Pirozynski, 1953). And in some cases, the decline in Q_{O_2} was recognizable but not large enough to explain the decline in weight-specific metabolic rate of intact animal (Elliott and Henderson, 1948; Krebs, 1950; Bertalanffy and Estwick, 1953; Bertalanffy and Pirozynski, 1953; Girard and Grima, 1980).

In fishes, works of this subject are limited to a few tissues for any given species and cover relatively small ranges of body size, and the results are not consistent. The value of Q_{O_2} was reported to decrease with slopes of -0.145 in the liver of toadfish *Opsanus tau* of 80–600 g (Vernberg, 1954), -0.139 in the gills of cutthroat trout *Salmo clarki clarki* of 10–100 g and -0.148 in the kidney of the same specimens (Holmes and Stott, 1960). On the other hand, the value of Q_{O_2} was reported to increase with increasing body mass with slopes of 0.202 in the brain of above-mentioned toadfish, 0.005 (calculated by us based on the data of Vernberg, 1954) in muscle of the same fish, and 0.15 in the brain of scup *Stenotomus chrysops* of 80–500 g (Vernberg, 1954). Increases in Q_{O_2} with increasing body mass were observed also in the gills of bluegill *Lepomis macrochirus* of 4–130 g and pumpkinseed *L. gibbosus* of 4–110 g (O'Hara, 1971).

We present, in this paper, the data on Q_{O_2} of 11 different tissues composing the main parts of the body of a freshwater teleost, carp *Cyprinus carpio*, ranging from 0.5 to 1100 g as well as the slope of each line showing the linear relationship in the log–log plot of Q_{O_2} of the tissues to the body mass. We also present the results of determination of Q_{O_2} value of the whole body which was prepared in the same way as in tissues, with carp ranging from 0.0016 to 0.8 g, and show the relationship between the Q_{O_2} value of the whole body and body mass.

MATERIALS AND METHODS

Fish used

The experiments on Q_{O_2} of various tissues were carried out on 212 carp *Cyprinus carpio* of 0.5–1100 g in fresh weight. The experiments on Q_{O_2} value of the whole body were carried out on about 600 carp of 0.0016–0.8 g in fresh weight. The fish smaller than 30 g were raised in our laboratory feeding on live water fleas *Daphnia pulex* for a week after two days old and marketed carp diet made from fish meal, wheat flour, soybean cake, vitamins and minerals as well as live water fleas after nine days old. Water temperature was kept at 19.5–20.5°C. The fish of 30–1100 g were raised at a commercial fish farm and transported to our laboratory. They were acclimated at 19.5–20.5°C for more than two weeks feeding on the marketed carp diet. Fish were not fed the day just prior to experimentation. One fish was used for each determination in most cases, but several fish for each determination in cases of very small fish.

Preparation of tissues

For an experiment on Q_{O_2} of various tissues, a fish (several fish in the case of very small fish) was instantly killed by a cut on the spinal cord immediately posterior to the head, and promptly dissected for excising tissues. The excised tissue was

washed in Cortland saline (Wolf, 1963) chilled with ice to remove blood and contaminants, and prepared for Q_{O_2} determination by the methods which proved, in a previous study (Oikawa and Itazawa, 1983), to give the most reliable results. Kidney, brain, intestine, hepatopancreas, spleen, gonads and muscle (the dorsal lateral muscle, but the dorsal lateral muscle mixed with the dorsal carinal muscle in the case of very small fish) were prepared by chopping about 400 times with ophthalmic scissors in a chilled weighing bottle until the tissue became pasty. Gill filaments, pectoral fin, caudal fin and dorsal fin were examined intact or virtually without any treatment.

For an experiment on Q_{O_2} of the whole body, fish of sufficient number to get tissue preparation of about 0.2 g were immobilized in chilled Cortland saline. The immobilized fish were blotted with a filter paper to remove saline, and then chopped following the above-mentioned method.

Substrate solution

Cortland saline with addition of substrates was used as the basic medium. The medium consisted of NaCl 7.23 g, KCl 0.41 g, $MgSO_4 \cdot 7H_2O$ 0.255 g, $NaHCO_3$ 0.333 g, glucose 1.45 g, Na-pyruvate 0.472 g, Na-fumarate 0.714 g, Na-L-glutamate 0.802 g and deionized water 1000 ml. The solution to suspend tissue preparation was made by adding two parts of 0.0905 M $CaCl_2$ and 10 parts of 0.1 M Na-phosphate buffer consisting of 8.7 parts of 0.1 M Na_2HPO_4 and 1.3 parts of 0.1 M NaH_2PO_4, in this order, to 112 parts of the basic medium. The solution was 0.58°C in depression of freezing point and 7.8 in pH value.

Determination of Q_{O_2}

Value of Q_{O_2} was determined at 20°C by a Warburg manometer shaking 120 times/min, using 20% KOH solu-

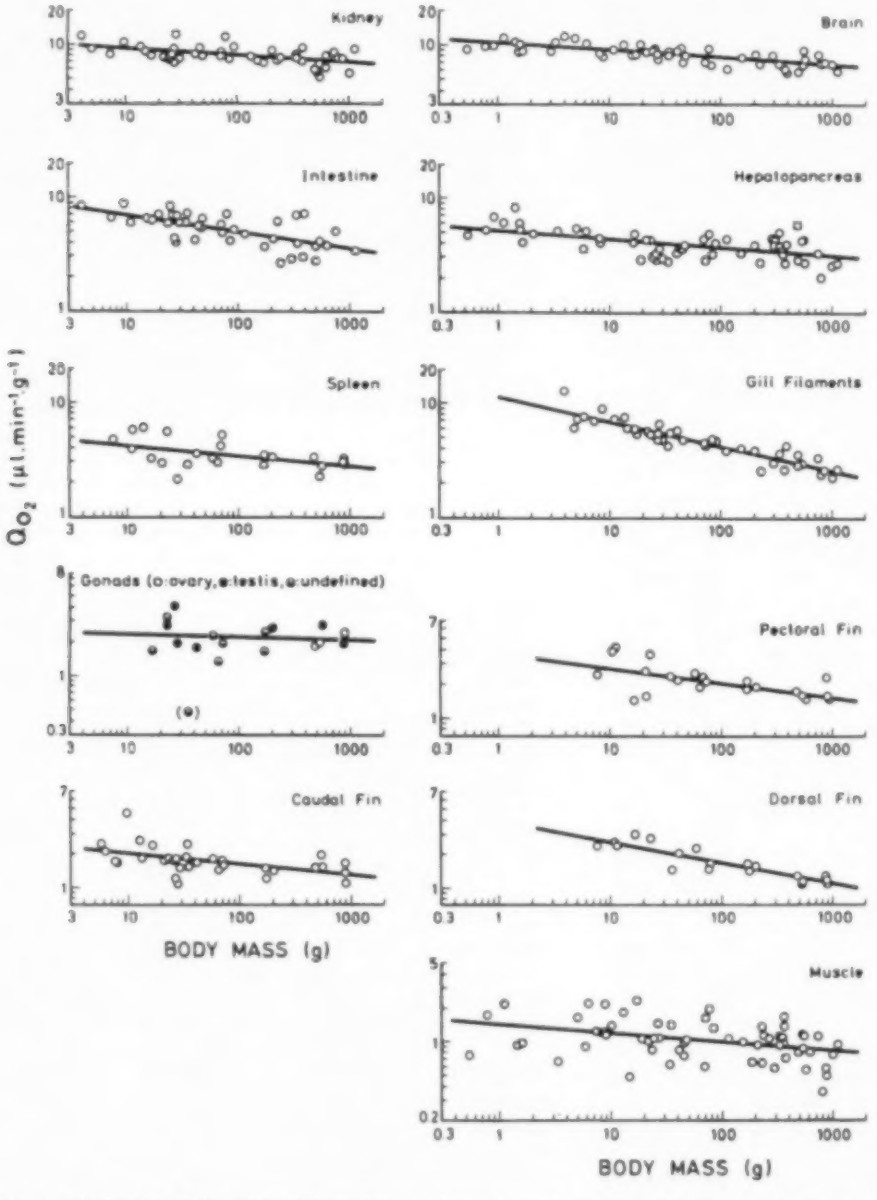

Fig. 1. Relationship of Q_{O_2} of 11 different tissues of carp to the body mass. For the gonads, the value in parenthesis was omitted from the calculation of *c* and *d*.

tion as CO_2 absorbent and ordinary air as the gas phase. Time from killing a fish to setting a tissue preparation in the reaction chamber was less than 30 min, and the tissue sample was kept chilled for the period. The manometer was read every 5 min for 60 min after thermal equilibration for 10 min, and the value of Q_{O_2} was determined by applying the least square method to the results for the first 30 min which showed a linear relationship to time.

RESULTS

The relationship between Q_{O_2} of various tissues and body mass

The values of Q_{O_2} (μl/min/g fresh wt) showed, in the log–log plot, a linear relationship to body mass (g/fresh wt) in all of 11 tissues examined (Fig. 1). The relationship between Q_{O_2} and body mass was expressed by an allometric equation, $Q_{O_2} = cW^d$, where c and d were constants. The constant c shows the level of metabolic activity of a tissue excised from a fish of 1 g in body mass. The value of Q_{O_2} compared at 1000 g level of body mass was the highest in the kidney (7.13) and the brain (6.95), while the lowest in muscle (0.835) and fins (1.10–1.57), being arranged in the order of kidney, brain, intestine, hepatopancreas, spleen, gill filaments, gonads, pectoral fin, caudal fin, dorsal fin and muscle (Table 1). The values of r in Table 1 show the correlation coefficients between log Q_{O_2} and log W. A negative value of the exponent d means that the value of Q_{O_2} declines with growth, and the larger the absolute value of d is, the steeper the slope is. The value of d was negative in all of 11 tissues examined, and the negative correlation was statistically significant based on t-test ($0.01 < P < 0.05$ in spleen, $0.001 < P < 0.01$ in pectoral fin, caudal fin and muscle, $P < 0.001$ in kidney, brain, intestine, hepatopancreas, gill filaments and dorsal fin) except the gonads. The largest absolute value of d was observed in gill filaments (-0.214) while the smallest in gonads (-0.0201) of which the value of Q_{O_2} was virtually constant independently of body mass.

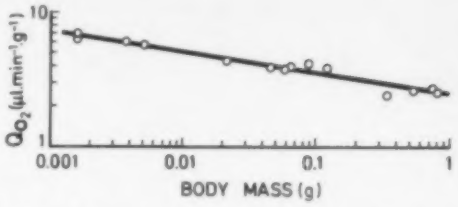

Fig. 2. Relationship of Q_{O_2} value of the whole body of carp to the body mass. $Q_{O_2} = 2.47\,W^{-0.153}$ (number of determinations was 14, and the correlation coefficient between log Q_{O_2} and log W was -0.969).

The relationship between Q_{O_2} value of the whole body and body mass

The values of Q_{O_2} of the whole body also showed, in the log–log plot, a negative correlation to body mass, and the negative correlation was statistically significant based on t-test ($P < 0.001$, Fig. 2). The relationship was expressed by an allometric equation, Q_{O_2} of the whole body (μl/min/g) $= 2.47W^{-0.153}$, or another allometric equation, total oxygen consumption *in vitro* (μl/min/fish) $= 2.47W^{0.847}$.

DISCUSSION

The value of Q_{O_2} of carp tissues showed negative correlations with body mass (W) expressed by an equation, $Q_{O_2} = cW^d$, where d was a negative. This equation is of the same pattern with the equation for the decline in weight-specific metabolic rate (M/W) of intact animal with increasing body mass, $M/W = aW^{b-1}$, where $(b-1)$ is a negative. The values of d were, however, not approximate, in most tissues, to the value of $(b-1)$ which has been reported to be -0.16 in carp ranging from 0.0032 to 3500 g (Winberg, 1956). Only dorsal fin ($d = -0.181$) and intestine (-0.144)

Table 1. Metabolic rate of carp tissues (Q_{O_2}) with values of c and d in the allometric equation, $Q_{O_2} = cW^d$, where W is the body mass (g).

Tissue	N	Q_{O_2} (μl.min^{-1}.g^{-1}) at body mass (g) of: 1 (value of c)	1,000	d	r
Kidney	44	10.60	7.13	-0.0575	-0.500
Brain	51	10.49	6.95	-0.0596	-0.729
Intestine	41	9.72	3.59	-0.144	-0.649
Hepatopancreas	57	5.26	3.15	-0.0741	-0.577
Spleen	23	5.21	2.76	-0.0919	-0.519
Gill filaments	41	11.41	2.60	-0.214	-0.924
Gonads	18	2.38	2.07	-0.0201	-0.108
Pectoral fin	22	3.59	1.57	-0.120	-0.604
Caudal fin	32	2.46	1.33	-0.0890	-0.491
Dorsal fin	19	3.85	1.10	-0.181	-0.896
Muscle	62	1.40	0.835	-0.0748	-0.381

showed d values relatively close to -0.16. Many tissues, spleen (-0.0919), caudal fin (-0.089), muscle (-0.0748), hepatopancreas (-0.0741), brain (-0.0596), kidney (-0.0575) and gonads (-0.0201) gave much smaller absolute values of d than -0.16 with significant differences based on t-test ($P < 0.05$) to -0.16, except spleen ($P = 0.052$) (Table 1).

This means that Q_{O_2} of most tissues of carp declines with increasing body mass much less steeply in comparison with weight-specific metabolic rate of intact carp. This means, in other words, that the decline in weight-specific metabolic rate is not explained solely by the decline in tissue respiration. These conclusions coincide with those in mammals in which the slope was reported to be -0.064 in kidney cortex, -0.069 in brain cortex, -0.095 in lung, -0.115 in liver and -0.139 in spleen of mouse, rat, guinea-pig, rabbit, cat, dog, sheep, cattle and horse (Krebs, 1950, quoted from Bertalanffy and Pirozynski, 1953), -0.065 in skeletal muscle of rat (Bertalanffy and Estwick, 1953), and -0.05 in heart, -0.085 in lung and -0.018 to -0.116 in liver of rat of various size ranges (Bertalanffy and Pirozynski, 1953).

The Q_{O_2} value of the whole body decreased, on the other hand, with increasing body mass at a slope of -0.153 which was very close to the above-mentioned slope for oxygen consumption of intact carp, -0.16 (Winberg, 1956). This implies that the decline in metabolic rate with increasing body mass exists *in vitro* in the same extent as *in vivo*. We have proposed an idea that the decline in weight-specific metabolic rate of intact animal with increasing body mass "could be explained, partly at least, if tissues with low metabolic rates get larger with growth in weight in proportion to the whole body" (Itazawa and Oikawa, 1983). The present results in the Q_{O_2} of tissues and the Q_{O_2} value of the whole body are considered to partly give a support to our above-mentioned idea. We are now carrying out examinations of this idea, based on the present results in Q_{O_2} value of various tissues at various body sizes as well as figures for the relative growth of various tissues and organs.

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THE VISCOSITY CHANGE OF THE BODY-WALL DERMIS OF THE SEA CUCUMBER *STICHOPUS JAPONICUS* CAUSED BY MECHANICAL AND CHEMICAL STIMULATION

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Abstract—1. Normal viscosity of the body-wall dermis of *Stichopus japonicus* Selenka ranged from 0.08 to 35 MPa · sec. The mean was 1.1 MPa · sec.
2. Mechanical stimulation doubled the viscosity.
3. Artificial seawater with elevated potassium concentration reversibly increased the viscosity three times.
4. Acetylcholine (10^{-5} M) increased the viscosity soon after the application, and later it decreased the viscosity to half of that before application.
5. Coelomic fluid of *S. japonicus* increased the viscosity reversibly. The increase was greater in the dermis with lower viscosity.

INTRODUCTION

The body-wall dermis of sea cucumbers rapidly changes its viscosity in response to stimulation. This rapid viscosity change helps control of the tone of the body (Motokawa, 1981, 1982a). Because the dermis is a collagenous connective tissue with no or little muscle (Motokawa, 1982b), the viscosity change has been attributed to the viscosity change in the extracellular materials (Motokawa, 1981; Eylers, 1982). Although it is necessary to study the chemistry of the dermis to understand the mechanism of the change, there have appeared only few brief reports on the chemical composition of the body wall of sea cucumbers (Giese, 1966; Prim *et al.*, 1976). *Stichopus japonicus* is an exception. The body-wall chemistry of this species was intensively studied by Tanikawa (1955). Information on the chemical and morphological features of collagen in the dermis is also available for this species (Matsumura *et al.*, 1973; Matsumura, 1974). Therefore *Stichopus japonicus* is best suited for the study of the mechanism of the viscosity change of the body-wall dermis. The reason why this species has been intensively studied is that this species has been an important marine product in Japan. The methods of propagation and various other aspects of biology have been studied on this species (Sang, 1963). Here I report the rapid and reversible change in viscosity of the body wall of this important species.

MATERIALS AND METHODS

Specimens of *Stichopus japonicus* were obtained from Jogashima Fishermen's Union, Miura, Kanagawa Prefecture. The relaxed body length of the animal was 15–20 cm. The animals used were Akanamako or red sea cucumber, which is one of three forms of this species (Sang, 1963). The experiments were performed during the months December–June. The Akanamako is known to be active during these months (Mitsukuri, 1903).

Creep tests were performed to measure the viscosity of the dermis. A column of a dermis of *ca.* 4 × 4 × 25 mm size was cut out from the dorsal body wall. The long axis of the dermis sample, along which a load was applied, coincided with the circumferential axis of the animal. The sample was fixed to the bottom of the experimental trough, which was filled with 50 ml artificial seawater. The other side of the sample was glued to a holder with a cyanoacrylate glue, and the holder was connected to a lever. A load was applied on the dermis through the lever, and the resulting elongation was recorded kymographically. The stress in the dermis at the beginning of the experiment was 0.3–3 kPa. The dermis rapidly elongated at first, but later it showed more or less constant rate of elongation (see Results). The stress divided by this constant rate of elongation gives the normal viscosity of the dermis (Motokawa, 1982a). The effect of stimulation was described by the relative viscosity: it is defined as the normal viscosity after the stimulation which is normalized by the normal viscosity just before the stimulation.

All the experiments were carried out in artificial seawater (ASW). The composition of ASW was (in mM): NaCl, 433.7; KCl, 10.0; $CaCl_2$, 10.1; $MgCl_2$, 52.5; $NaHCO_3$, 2.5 (pH 8.1–8.2). In artificial seawater with 100 mM potassium concentration (KASW), the sodium concentration was decreased so as to keep the osmotic concentration constant.

The coelomic fluid (CF) of *Stichopus japonicus* was prepared as follows. An incision was made in the body wall of *S. japonicus* and CF was collected as it flowed out. The pH of the CF was adjusted to 8.1–8.2 with a few drops of 0.1 N NaOH and the CF was diluted with ASW to a final concentration of either 1/2 (0.5 CF), 1/10 (0.1 CF), or 1/100 (0.01 CF) of the original CF.

All the experiments were carried out at room temperature (18–22°C). The temperature did not vary more than 1°C in a single creep test.

RESULTS

Creep curve and normal viscosity

Figure 1 shows how the dermis elongates under a load. The dermis elongated rapidly at first, and then the rate of elongation or the creep rate decreased to a

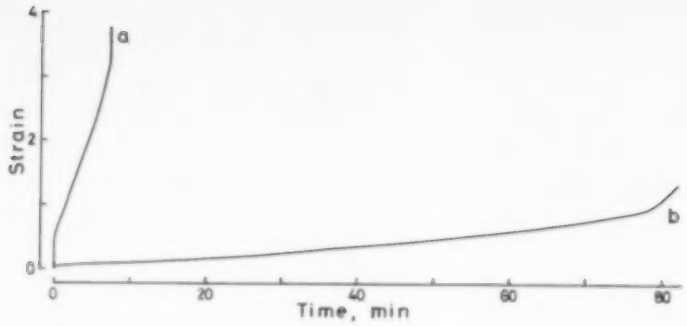

Fig. 1. Extension curves of two dermis samples of *Stichopus japonicus* under a constant load at 19°C. The dermis broke at the end of the curves. The stress at the beginning of the experiment was 2.5 kPa. The normal viscosity calculated from the curves are 0.54 MPa·sec (a) and 35 MPa·sec (b). Strain is defined as elongation/initial length.

constant value. The dermis continued elongation at this rate. Then the rate gradually increased again and finally the dermis ruptured. This creep behaviour roughly coincides with that of a four-element mechanical model which consists of a Maxwell element and a Voigt element connected in series (Goto *et al.*, 1962). The instantaneous elongation of the dermis at the application of the load corresponds to the extension of the spring of the Maxwell element. The phase of gradually decreasing rate corresponds to the retarded extension of the Voigt element. The phase of the elongation with a constant rate corresponds to the continuous flow of the dashpot of the Maxwell element. The dermis had the last phase with gradually increasing rate, which is different from the creep behaviour of the model. The discrepancy will be explained as follows: as the dermis elongates, the cross sectional area decreases and thus the stress increases, which increases the rate of elongation. This explanation is supported by the observation that the narrowing or necking of the dermis was always observed when the dermis approached the breaking strain.

The viscosity of the dashpot of the Maxwell element, or the viscosity which lies in series with the applied force, is calculated from the constant flow rate. This viscosity is generally called the normal viscosity (Nakagawa, 1978). The normal viscosity of the dermis showed great variation. Figure 1 compares the creep curve of the dermis with rather small normal viscosity (curve a) with that of the dermis with quite large viscosity (curve b). Figure 2 shows frequency distribution of the normal viscosity of 72 dermis samples taken from 23 animals. The minimum is 0.076 MPa·sec and the maximum is 35 MPa·sec, which is 450 times greater than the minimum. The median is 0.80 MPa·sec. The geometric mean is 1.1 MPa·sec (SD of logarithmically converted value: 0.6). The arithmetic mean is 3.0 ± 5.6 MPa·sec ($\pm$SD). The samples taken from the same individual animal showed different normal viscosities. For example, a dermis piece of $16 \times 4 \times 25$ mm size was divided into four pieces of equal size. The normal viscosities of these pieces were 4.4, 0.1, 0.3 and 1.5 MPa·sec respectively. The wide variation of the normal viscosity is quite likely a reflection of the ability of a dermis to change viscosity, as will be shown in the next section.

Effect of stimulation

The creep curve suggests that the mechanical properties of the dermis of *S. japonicus* are roughly simulated by a four-element mechanical model. I have studied the mechanical properties of the dermis of two other species of sea cucumbers. The dermis of these species were well simulated by the same mechanical model as used in the present study. Chemical stimulation caused changes in the viscosity of dashpots of the model but caused little change in the elasticity of springs (Motokawa, 1984). In this section the elasticity of the dermis is assumed to be constant.

The mechanical stimulation transiently increased

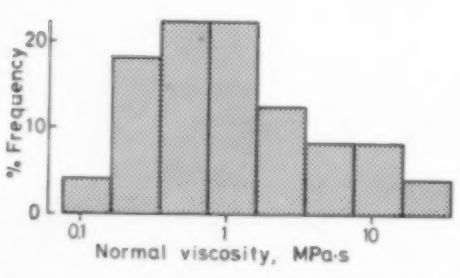

Fig. 2. Frequency distribution of the normal viscosity of the dermis in artificial seawater ($N = 72$).

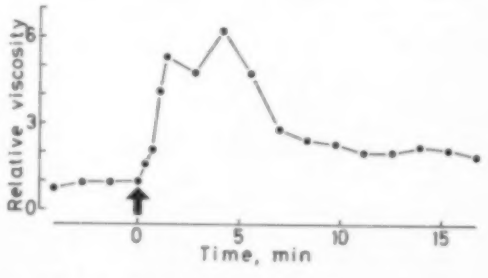

Fig. 3. Effect of mechanical stimulation on the normal viscosity of the dermis. The dermis was gently squeezed with forceps at the time indicated by an arrow.

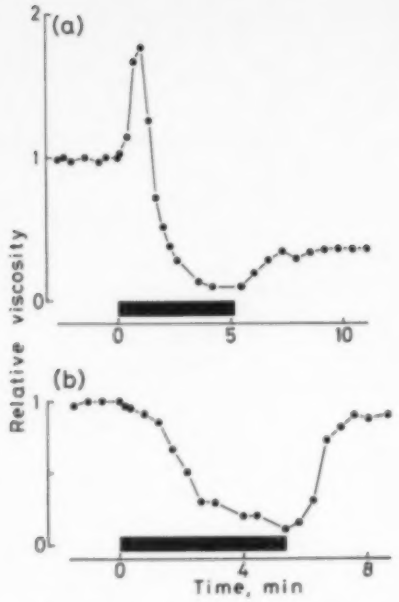

Fig. 4. Effect of acetylcholine (10^{-5} M) on the normal viscosity of the dermis. Bars indicate the period of application of ACh. In most cases ACh caused a rapid increase in viscosity which was followed by a decrease in viscosity (a). In some cases viscosity decrease alone was observed (b).

the normal viscosity (Fig. 3). The average of the peak viscosity is given in Table 1.

Acetylcholine (ACh) at the concentration of 10^{-5} M increased the normal viscosity for 1–2 min and after that it decreased the viscosity less than that before application of ACh (Fig. 4a). Washing out of ACh increased the viscosity again. The amount of initial increase in viscosity varied with dermis samples. In two samples out of 16, no initial increase was observed, and only the decrease of the viscosity was prominent (Fig. 4b). The viscosities at 1 and 5 min immersion in ACh are given in Table 1.

KASW reversibly increased the viscosity (Fig. 5). The viscosity in KASW for 3 min is given in Table 1.

Coelomic fluid of *S. japonicus* reversibly increased the normal viscosity (Fig. 6). The 0.01 CF was still

Table 1. Effect of mechanical and chemical stimulation on the relative viscosity of the body-wall dermis of *Stichopus japonicus*

Stimulation	Minutes after stimulation*	Relative viscosity average ± SD (*N*)
Mechanical stimulation	1–5†	2.63 ± 1.51 (11)
Acetylcholine (10^{-5} M)	1	1.74 ± 0.82 (16)
	5	0.62 ± 0.38 (16)
Potassium-rich (100 mM) artificial seawater	3	3.02 ± 1.20 (10)
Coelomic fluid, 0.5 CF	3	2.60 ± 1.27 (25)
0.1 CF	3	2.33 ± 1.31 (7)
0.01 CF	3	1.32 ± 0.28 (5)

*The time when the viscosity was measured.
†The peak value during this period was measured.

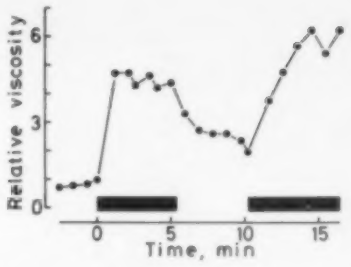

Fig. 5. Effect of potassium rich (100 mM) artificial seawater on the normal viscosity of the dermis. A bar indicates the period of application of KASW.

slightly effective (Table 1). The effect was dependent on the normal viscosity of the dermis before the application of CF. The viscosity increase was greater in the dermis with less normal viscosity (Fig. 7). Figure 7 also shows that the effect of CF varied much even when CFs were applied to the dermis of similar normal viscosities. Because CFs prepared from different individual animals had different activities, the variation in Fig. 7 at the same normal viscosity is probably due to this difference. For example, the 0.5 CF prepared from one animal caused a more than three-fold increase in viscosity in three dermis samples with the normal viscosity 0.9–1.3 MPa·sec. The 0.5 CF from another animal caused at most a 1.4 times increase in viscosity in three dermis samples with normal viscosity 0.2–1.5 MPa·sec.

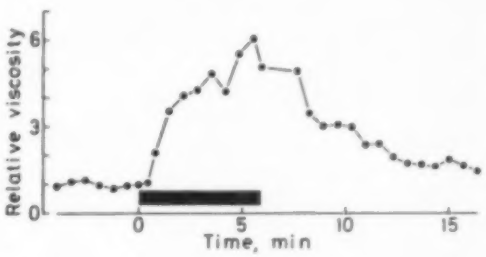

Fig. 6. Effect of coelomic fluid (0.5 CF) on the normal viscosity of the dermis. A bar indicates the period of application of CF.

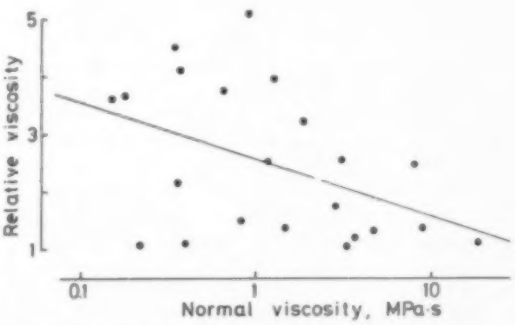

Fig. 7. The relation between the normal viscosity before stimulation (abscissa) and that of after stimulation by 0.5 CF (ordinate). The regression line, $y = 2.58 - 0.99 \log x$, is significant at 5% level ($r = 0.45$).

The increased normal viscosity caused by chemical or mechanical stimulation did not exceed 35 MPa · sec, the maximum normal viscosity observed in ASW.

DISCUSSION

The dermis of *Stichopus japonicus* changed the rate of elongation under a constant load in response to mechanical and chemical stimulation. The result that the effect of CF was dependent on the normal viscosity before stimulation strongly suggests that the stimulation changes the normal viscosity, not elasticity, as in other sea cucumbers (Motokawa, 1982a, 1984). These results also suggest that the wide variation of the normal viscosity in ASW reflects the ability of the dermis to change the normal viscosity. In conclusion, the dermis of *S. japonicus* in ASW will either be in the "soft" state with the normal viscosity about 0.1 MPa · sec, in the "intermediate" state with the normal viscosity about 1 MPa · sec, or in the "stiff" state with the normal viscosity about 10 MPa · sec. The stimulation increases or decreases the viscosity. The "softer" dermis responds more to the "stiffening" stimuli such as CF, KASW and the mechanical stimulation.

The increase in "stiffness" of the holothurian body wall caused by mechanical stimulation has been reported by several authors (Uexküll, 1926; Serra-von Buddenbrock, 1963). Stott *et al.* (1974) and Freinkel and Hepburn (1975) reported that the mechanical stimulation increased the elastic stiffness of the body wall. The increase in the elastic stiffness depended on the prestimulation stiffness. The apparent change in elasticity is likely to be caused by the change in the viscosity, because the strain rate they used seems to be slow compared with the relaxation time of the dermis.

The effects of chemical stimulation were similar to those reported for another sea cucumber *Stichopus chloronotus* (Motokawa, 1981, 1982a). As acetylcholine was effective, the viscosity of the dermis is quite likely to be controlled by nerves. ACh increased the viscosity at first and then decreased it. Motokawa (1982a, c) found that the CF contained both "stiffening" and "softening" chemical factors. These observations suggest that the nervous control is bidirectional: there are two types of nerves, one "stiffens" and the other "softens" the dermis.

The chemical composition of the body-wall dermis of *S. japonicus* was studied by Tanikawa and co-workers (Tanikawa, 1955; Tanikawa *et al.*, 1955; Tanikawa and Yoshitani, 1955). Water content is 89% of wet weight. Levels of protein, carbohydrate and lipid (in percent dry weight of the dermis) are 40, 5–8 and 8% respectively. Collagen is 4% of total protein. The collagen of the body wall of *S. japonicus* was studied by Matsumura and co-workers (Matsumura, 1972, 1974; Matsumura *et al.*, 1973). Its chemical composition and shape are not markedly different from those of usual mammalian collagen. The body wall of *S. japonicus* contains both neutral- and acid-mucopolysaccharide-protein complex to the amount of 1% of dry weight (Tanikawa, 1955; Motohiro, 1960). Tanikawa (1955) studied the swelling of the dermis at various pH values. He found that the dermis remarkably swelled at pH 7–10. He attributed the swelling to the chemical characters of the mucoprotein in the dermis. pH has been reported to affect the mechanical properties of other echinoderm connective tissues, the mechanical properties of which are controlled by nerves (Takahashi, 1967; Maeda, 1978; Wilkie, 1978; Hidaka, 1983). A change in pH may be occurring in the holothurian dermis, thus changing the interactions between the molecules of the body wall, which results in the change in viscosity.

Stichopus japonicus crawls under rocks and becomes dormant during August and September. The body wall of the sea cucumber is rigid during this summer sleep (Mitsukuri, 1903). Probably the body wall is maintaining the "rigid" state through the same mechanism as that observed in the present study. The stiffness-controllable connective tissues are working for tone control in echinoderms and their role will be similar to the molluscan catch muscles (Motokawa, 1982a, 1983). The energy for maintaining the stiffness will be low, and thus enabling the animal to "sleep" safely in a stiff state with little expenditure of energy during the hot season.

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SALINITY TOLERANCE AND SODIUM BALANCE IN THE PRAWN *PALAEMONETES PUGIO* HOLTHUIS, IN RELATION TO OTHER *PALAEMONETES* SPP.

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(*Received* 28 *June* 83)

Abstract—1. Determined lethal limits (96-hr LD_{50} values) for *Palaemonetes pugio* adults are 0.5 and 44‰; for larvae (first zoeae), 16 and 46‰.

2. The concentration of blood sodium remains relatively constant over the salinity range 1–40‰.

3. In relation to other species of *Palaemonetes*, it is suggested that *P. pugio* represents an intermediate stage in transition from salt to fresh water, the species being tolerant of very low salinity and able to regulate blood sodium over a wide range as adults, but requiring brackish to marine water as recently hatched larvae.

INTRODUCTION

Prawns of the genus *Palaemonetes* are widely distributed throughout the world, being especially common in aquatic habitats of Europe as well as North America. The mature adult is rather small (about 2–3 cm total length) and is characterized by a relatively transparent exoskeleton. As in most carideans, *Palaemonetes* embryos develop within eggs brooded on the pleopods of the female, then hatch and become planktonic zoea larvae, which undergo several moults and changes of form until metamorphosis (see Broad, 1957; Broad and Hubschman, 1962).

Some species of "grass shrimps" are marine or estuarine; others are found in fresh water. Observed distribution patterns of *Palaemonetes* spp. in relation to salinity may be accounted for by their relative ability to tolerate a particular range of salinity (and extent of change over this range) and their ability to maintain internal water and salt balance, at different stages (e.g. larva, juvenile, adult) in the life cycle.

Some information on salinity tolerance and osmotic/ionic regulation in *Palaemonetes* spp. has been gleaned from laboratory and field investigations (see Discussion for references) but many gaps remain. For example, data on life-cycle stages other than the adult are rudimentary. Once determined, salinity relations may be used to ascertain dispersal capabilities of particular species and, along with morphologic and developmental characteristics, to deduce possible evolutionary pathways taken by palaemonids in their invasion of assorted aquatic habitats (Strenth, 1976).

Palaemonetes pugio, the object of the present study, is a dominant and ecologically important species in estuaries and tidal marshes of the eastern United States (Welsh, 1975) ranging from Maine to Texas (Knowlton, 1973; Williams, 1974) yet little is known about salinity requirements of adults or larvae, except for notation of attendant salinity conditions where particular species (as adults mainly) have been collected, and some survival data from laboratory studies. In coastal North Carolina, *P. pugio* has been found in media ranging from 0 to 35‰ (Knowlton, 1970; Knowlton and Williams, 1970; Thorp and Hoss, 1975). In the Chesapeake estuarine system, it has been collected in water of 0‰ (Bowler and Seidenberg, 1971) to 39.8‰ (Yost, 1980) salinity. Thorp and Hoss (1975) measured survival of animals held for 21 days under three salinity (5, 20, 35‰) and temperature regimes, observing highest survival at 20‰, lowest at 5‰. Compared with *P. vulgaris*, Bowler and Seidenberg (1971) observed greater tolerance of low test salinities (1, 2 and 3‰) by *P. pugio*, and less tolerance of high salinities (36 and 40‰). In an experiment testing effects of salinity and temperature on survival and growth of postlarvae and juveniles (from Galveston Bay, Texas) Wood (1967) noted best survival (over 30 days) within the 4–16‰ range. Under laboratory conditions, considerable mortality of *P. pugio* zoeae in salinities below 10‰ has been observed (Broad, 1957; Middaugh and Floyd, 1978; McKenney and Neff, 1979).

While it has been established that *P. pugio* adults are euryhaline and larvae less so, investigation of osmotic and ionic regulation in this species has been initiated only recently (Kirby and Knowlton, 1976; Roesijadi *et al.*, 1976a,b; Lucu, 1977; Lucu *et al.*, 1977). The extent to which a species regulates is indicated by measuring concentrations of major ions, such as sodium, in the blood (hemolymph) in relation to amounts in surrounding water.

The objectives of our research project were: (1) to determine lower and upper median lethal limits (LD_{50}, LC_{50} or TL_m i.e. dosage or concentration of salinity at which half of a population of test animals dies within a particular time interval) of *P. pugio*

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larvae as well as adults, and (2) to indicate regulatory ability by measuring sodium concentration in the blood of the adult over a wide range of external salinity. In addition, results obtained for *P. pugio* were compared with similar data pertaining to other species within the genus. In this comparative study, we attempted to determine, in as many species as possible, whether the animals are stenohaline or euryhaline, to correlate this with habitat, and to draw conclusions concerning degree of specialization with regard to osmotic physiology.

MATERIALS AND METHODS

Specimens of *P. pugio* were collected from a tidal pool of the Patuxent River estuary near Benedict, Maryland, USA. Salinity was approximately 16‰ at time of collection. In the laboratory, animals were transferred from the collecting container to an aquarium containing artificial seawater ("Instant Ocean" sea salts added to distilled water) of salinity equal to that of their habitat; adults were kept there for a minimum of four days before being used in experiments. Test solutions of synthetic seawater were made by diluting a stock solution of 70‰ to the desired salinity as measured by hydrometer.

In determination of lethal limits of salinity, adult animals were exposed to seventeen concentrations of artificial seawater ranging from 0 to 70‰ salinity (Table 1). Recently hatched (Form I) zoea larvae were also tested (beginning a few hours after hatching) at eight salinities throughout the same range (Table 2). Animals were placed into plastic compartmented trays with lids closed, each compartment containing one animal and 25 ml of aerated test water of known salinity, maintained at room temperature (23 ± 3°C). Any deaths that occurred were noted frequently until such time (up to 154 hr) that adaptation to the change was indicated (by drastic reduction in frequency of deaths). Cessation of scaphognathite (gill bailer) movement was taken as the criterion for death. Inasmuch as tolerance is affected by moulting (in *Palaemon ritteri*; Reynolds, 1975) recently moulted animals were not employed in the tests.

In order to measure blood (hemolymph) sodium concentration as a function of external salinity, groups of adults were exposed to one of ten test solutions, as follows: 1, 6.8, 12, 15, 22, 27.8, 29.3, 36, 37.3 and 40‰. In this study salinities were estimated by hydrometer and then pinpointed with a Coleman Flame Photometer Model 21 used in conjunction with a Coleman Junior II Spectrophotometer. Animals were placed in test solutions and allowed to equilibrate for 48 hr before their blood sodium was measured. Blood was collected with a microsyringe, the needle being inserted under the posteriodorsal edge of the carapace and directly into the heart. Blood samples ranged from 1 to 3 μl, depending on the size of the animal. In most cases 1 μl of blood was diluted to 3 ml with distilled water prior to photometry (at test salinity 1‰, 2 μl blood samples were diluted to 4 ml). Sodium concentrations of blood, as well as the test medium, were read from the flame photometer after it had been zeroed at the low end with distilled water and at the high end with 250 μM standard.

Table 1. Mortality of *Palaemonetes pugio* adults after 96 hr, and average time of death at various salinities

Salinity (‰)	No. of animals	Mortality (%)	Mean time of death (hr)
0	25*	100.0	8
1	36*	2.8	—
2.5	28*	0.0	—
5	43*	0.0	—
10	45*	2.2	—
20	27*	0.0	—
25	15	0.0	—
27	10	0.0	—
35	25*	8.0	—
40	20	30.0	13
47.5	20	70.0	21
50	30	83.3	19
55	35	68.6	33
57.5	10	100.0	4
60	5	100.0	2
63	10	100.0	2
70	15*	100.0	2

Mean death time values are based on a minimum of five individuals. Values marked with an asterisk (*) are pooled data from two or more test runs.

Table 2. Mortality of *Palaemonetes pugio* larvae (first zoeae) after 96 hr, and average time of death at various salinities

Salinity (‰)	No. of animals	Mortality (%)	Mean time of death (hr)
0	15	100.0	2
10	30	96.7	18
20	30	23.3	28
25	30	3.3	—
35	30	16.7	65
45	30	46.7	61
60	28	100.0	3
70	15	100.0	2

Mean death time values are based on a minimum of five individuals.

RESULTS

Results of tolerance tests on *P. pugio* adults are presented in Table 1 and Fig. 1. The table shows that the adults can tolerate almost any salinity they would normally encounter in an estuary. Although all animals died at 0‰, there was almost no mortality between 1 and 35‰ (within 96 hr). Deaths occurred more rapidly above 57‰ than at 0‰, somewhat more slowly between 40 and 50‰. Most mortality took place within 24 hr, about 75% of all deaths occurring within 10 hr.

Figure 1 shows per cent mortality in relation to salinity, for determination of lethal limits. At the low end of the zone of tolearance there is a sharp threshold (100% mortality at 0‰, almost no mortality at 1‰); at the high end, death may occur over a wider range (35–57.5‰). The lower lethal limit

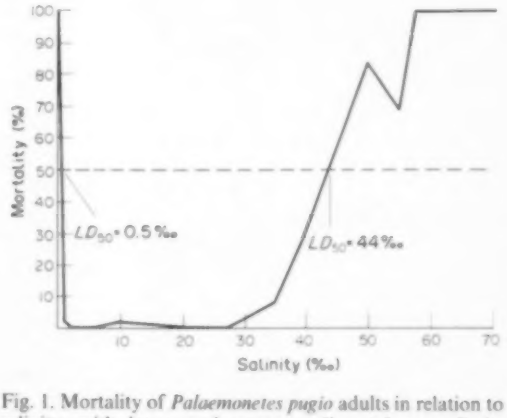

Fig. 1. Mortality of *Palaemonetes pugio* adults in relation to salinity, with lower and upper median tolerance limits (LD_{50}) indicated.

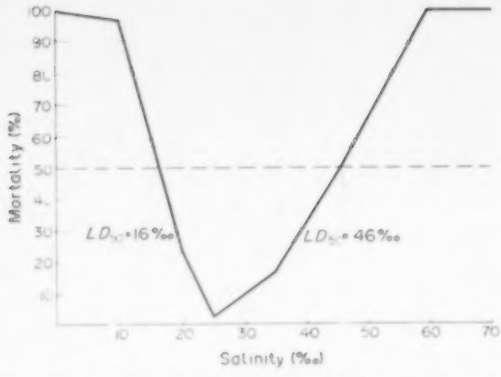

Fig. 2. Mortality of newly hatched *Palaemonetes pugio* larvae in relation to salinity, with lower and upper median tolerance limits (LD_{50}) indicated.

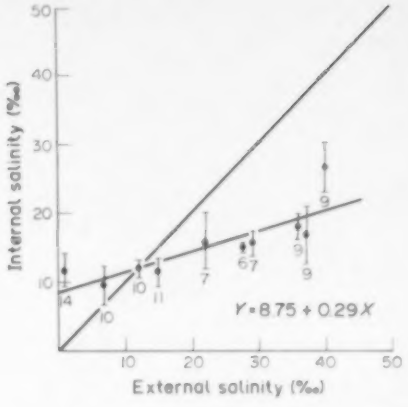

Fig. 3. Plot of sodium concentration, expressed as total salinity equivalent, in blood (internal) of *Palaemonetes pugio* vs that in medium (external). The mean value obtained at each (external) salinity is indicated by a solid circle, standard deviation by a vertical line; the number of samples tested is given under the vertical line. The diagonal line marks where internal and external media are isoionic.

(96-hr) is estimated at 0.5‰, and the upper limit is approximately 44‰.

Table 2 indicates that newly hatched *P. pugio* larvae cannot tolerate so wide a range of salinity as adults. Optimum larval survival is at 25‰, above the salinity in which they were hatched (16‰). At the salinity extremes the animals died much more rapidly than at the middle salinities.

Figure 2, a plot of per cent mortality vs salinity (after 96 hr of testing), shows an inverted bell-shaped curve slightly skewed toward survival at higher salinities. The lower 96-hr LD_{50} is estimated at 16‰, the higher one at 46‰.

Figure 3 shows that *P. pugio* adults are basically regulators of sodium, the blood being isoionic at about 12‰, hypoionic to the medium in higher salinities, and hyperionic in lower concentrations. The slope of the regression line is positive, indicating "slight" conformity—thus these animals are not "perfect" regulators. The values at 1 and 40‰ are off the line, possibly a result of lethal effects (inasmuch as these salinities are near the determined lethal limits).

DISCUSSION

In our experiments, mortality of *P. pugio* adults exposed to anionic (distilled) water was total and quick, yet there was virtually no mortality in salinities as low as 1‰. Notations (cited in Introduction) of presence of *P. pugio* populations in habitats where measured salinity was 0‰ suggest that adults can survive in fresh water, where ions presumably occur in trace amounts. Such survival is probably assisted by gradual acclimation to salt-deficient media. In view of the relatively high mortality (30%) that occurred at 40‰, natural populations would not be expected to occur in abundance above this level; indeed this is indicated by field data (Knowlton, 1970; Knowlton and Williams, 1970; Yost, 1980).

Maintenance of internal osmotic and chloride balance in *P. pugio* over a wide range of external salinity has been demonstrated by Roesijadi *et al.* (1976b). Similarly, Lucu *et al.* (1977) found that blood sodium levels between animals exposed to 2 and 17‰ (for 1 week) were about the same; those of animals in 32‰ were slightly (but significantly) higher. Our data, which portray measurements of blood sodium among animals exposed to ten different salinity levels between 1 and 40‰, are in agreement with those of Lucu *et al.* (1977) in that concentrations of this cation are seen to vary little among animals exposed to either hypo- or hyper-ionic media. However, at comparable salinities the sodium concentrations measured by them are somewhat higher, and their determined isoionic salinity is approximately 17‰ (vs 12‰; Fig. 3). A trend toward slight conformity, as delineated in our study (and shown by the positive regression line in Fig. 3) is also evident from their results, but only between 17 and 32‰.

There is some experimental evidence to indicate the mechanisms by which *P. pugio* is able to survive and regulate in hypo- and hyper-osmotic media. Water-turnover measurements by Roesijadi *et al.* (1976b) have demonstrated reduced permeability above and below the iso-osmotic salinity (17‰). Lucu (1977) determined sodium fluxes and electrochemical potentials in animals acclimated to hypo-, iso-, and hyperionic media, concluding that sodium efflux is diffusive in all media while chloride is actively extruded into seawater and is taken in from the dilute medium.

Previous studies of salinity tolerance and osmoregulation in adult *Palaemonetes* have been conducted on six other species besides *P. pugio* (Table 3). Parry and Potts (1965) studied balance in *P. antennarius*, a fresh water stenohaline species found in southern Europe, determining that this prawn maintains a level in the blood that is equivalent to about 40% seawater (=14‰ salinity*), and that its urine is almost iso-osmotic with the blood. This species shows an ability to take up sodium in dilute media

*In this paper, salt concentrations expressed elsewhere in other units are converted to ‰ salinity, for the sake of consistency, as well as greater ecological relevance. For interconversion, "average" (100%) seawater has a salinity of 34.3‰ and a sodium concentration of 470 mM (Prosser, 1973; Tables 2, 3, p. 84).

Table 3. Summary of principal salinity tolerance and osmoregulation studies on species of *Palaemonetes*

Species (and reference)	Collection site	Limits (and type) of tolerance	Osmoregulatory capability
P. antennarius (Parry and Potts, 1965)	River and spring (fresh water) near Verona, N. Italy	0.02–11‰ (oligostenohaline)	Oligohaline (Na taken up in dilute media only)
P. intermedius (Dobkin and Manning, 1964)	Coral Gables Waterway and Virginia Key, S. Florida, USA (brackish to marine)	5–39‰* (euryhaline)	Almost "ideal" (hypo- and hyper-) regulator
P. kadiakensis (Leinen, 1982)	Small ponds and drainage ditches, Missouri, USA (fresh water)	0–18‰† (stenohaline)	Osmoregulator in dilute media (Na and K taken up), osmoconformer above 23‰ (iso-osmotic point)
P. paludosus (Dobkin and Manning, 1964)	Fresh water rock pit S. Florida, USA	0–30‰* (euryhaline)	Osmoregulator in salinities less than 20‰, conformer in higher salinities
P. pugio (Knowlton and Kirby, this paper)	Patuxent River, Benedict, Maryland, USA (brackish)	1–55‰ (euryhaline)	Regulator (Na in blood maintained at nearly constant level)
P. varians (Potts and Parry, 1964)	Estuaries and marshes near Plymouth, UK (brackish)	0.2–42‰ (euryhaline)	Regulator (ion levels in blood maintained by uptake of Cl in dilute media, efflux of Na in concentrated media)
P. vulgaris (Schoen and Knowlton, 1977)	Sinepuxent Bay, Assateague Is., Maryland, USA (marine)	1–51‰ (euryhaline)	Regulator (Na in blood maintained at nearly constant level)

*No animals tested beyond these limits.
†70% mortality occurred at upper salinity, after 20 days.
Salinity limits expressed are those above and below which 100% mortality occurred, except where noted, in experiments.

(0.02–3.6‰), but death occurs within a few days above 11‰. It is said to be incompletely adapted to fresh water, since it must expend considerable energy to compensate for large salt losses by excretion (Parry, 1957). Dobkin and Manning (1964) investigated osmoregulation in two eastern American species: *P. intermedius*, a brackish-water dweller along the Atlantic coast, and *P. paludosus*, which inhabits fresh and slightly brackish water. They found that both species are euryhaline, and that they regulate blood ion levels in response to salinity change; *P. paludosus*, however, tends to conform at higher salinities. McGuire (1961) noted that the latter species dies within a day below 0.7‰, attributing the mortality to salt loss. Leinen (1982a,b) studied sodium regulation in *P. kadiakensis*, a freshwater species distributed west of the Alleghenies (U.S.A.). He determined that in this species internal sodium balance is maintained against a concentration gradient of 200:1 in deionized water as well as in its pondwater habitat, hypothesizing that sodium is obtained from food and is taken up at the body surface, primarily at the gills. Strenth (1976) tested salinity tolerance of *P. kadiakensis* adults progressively acclimated at 5‰ intervals for 2 hr each, to the final test salinity; from his data the 96-hr LD_{50} can be calculated for this species (25‰). Potts and Parry (1964) studied sodium and chloride balance in *P. varians*, a north-west European species that can tolerate 0.3–41‰ and is homoiosmotic (Panikkar, 1941). They found that in 22‰ (iso-osmotic salinity) this prawn is in passive equilibrium with the medium. In a more dilute medium, Na and Cl content of the blood is maintained mainly by active uptake of Cl, Na being held in equilibrium due to a potential difference produced by Cl uptake; in full-strength seawater Na is pumped out, the resulting negative potential maintaining Cl balance. Finally, Nagabhushanam (1960) examined tolerance of *P. vulgaris*, a marine species of the US Atlantic coast, to water of low salinity. Animals (from Woods Hole, Massachusetts) exposed to 0–2‰ survived no more than two days, while those in salinities above 3‰ survived the full 30-day test period. Schoen and Knowlton (1977) measured blood sodium in this species, finding that it remains relatively constant over the range 5–45‰.

The "zone of tolerance" for each of these six species (derived from data given in the aforementioned studies), plus that for *P. pugio*, is given in Table 3. In addition, each species is characterized with regard to normal habitat and osmoregulatory capability. It can be seen that the seven species represent a variety of niche adaptations at the physiological level, ranging from "conservative" to "freshwater-specialized". The more conservative species (assuming the Decapoda are of marine origin) would be orthostenohaline (see Kinne, 1971) since they would not be exposed to wide salinity fluctuations in an oceanic environment. Although *P. vulgaris* is not orthostenohaline, it is, more so than the other species listed, typically found in deeper waters of high salinity (Jenner, 1955; Knowlton and Williams, 1970), and consequently can be regarded as the most conservative of them, in terms of its relationship to salinity.

The family Palaemonidae is considered by some (Emery *et al.*, 1957) to be in the process of migration from marine to fresh water. Prawns in coastal areas

(i.e. estuaries where salinity is variable, and rivers where it is low) must have the ability to adjust to variable and/or low salinity, hence be euryhaline. *Palaemonetes intermedius*, *P. varians*, and *P. pugio*, being increasingly euryhaline (roughly in that order) and abundant in brackish (and *P. paludosus* in fresh) water, can be regarded as intermediates in this habitat transition. *Palaemonetes paludosus* seems to represent partial specialization toward life in fresh water, evidenced by "maintenance" of euryhalinity, and by greater similarity to marine decapods (than freshwater Crustacea) in terms of amino acid levels in muscle tissue (Turner *et al.*, 1975). *Palaemonetes antennarius* can be considered even more specialized physiologically, for it is both a freshwater inhabitant and oligostenohaline (Kinne, 1971). Regulation is more limited in the latter two species, further restricting them to freshwater habitats. *Palaemonetes kadiakensis*, too, is rather specialized for living in fresh water, not only because test populations of this species exhibit progressively higher mortality in water of higher salinity, but also because individuals are able to take up and conserve sodium very efficiently in extremely dilute media.

Distribution of the adult may be limited by responses of the larva to the environment. As determined in this study, *P. pugio* larvae are less tolerant of salinity than the adult. This is not surprising considering that the larva is an earlier stage in ontogeny, with osmoregulatory organs (gills, gut?) not fully developed. In addition, being smaller, larvae have a larger surface area to volume ratio, and are thus faced with relatively greater diffusive exchanges. Considering the whole life cycle, *P. pugio* seems to represent a "transitional" stage in adaptation to fresh water, the adult tolerating extremely low salinity (and able to regulate blood sodium over a wide range) yet presumably requiring at least brackish water for release and initial survival of larvae.

Results from our tolerance tests on larvae suggest that polyhaline to euhaline conditions are most conducive to survival while animals are in Form I. The optimum salinity, as described in these tests, is 25‰, which is close to that (21.7‰) calculated by McKenney and Neff (1979). There is some evidence however, that the larvae can acclimate to lower salinities. McKenney and Neff (1979) found that in a group of laboratory-reared zoeae acclimated to 8‰ for 3 hr, then exposed to 3‰ (and 25 or 30°C) thereafter, about half survived to metamorphosis. Also, individuals have been collected from waters of low salinity. Herman *et al.* (1968) captured *P. pugio* larvae in zooplankton of the Patuxent River estuary in water of 19.2‰ or lower, but they did not specify salinity conditions where zoeae were found. Larvae belonging to the genus *Palaemonetes* have been taken over a broad salinity range—1.76 to 32.34‰ in the York River and lower Chesapeake Bay, Virginia (Sandifer, 1973), 0.5 to 36‰ along the North Carolina coast (Knowlton, 1970)— but the species captured may not have been *P. pugio* and, in the latter case at least, the animals taken were mostly zoeae in later stages of development. Knowlton (1970) noted that numbers of *Palaemonetes* larvae were greatest in the range 25–35‰, decreasing in lower salinities.

Robertson (1960) calls attention to two categories of freshwater Crustacea: those that must return to the sea to breed, and those that live and breed in fresh water. Among the Palaemonidae, *P. pugio* can be considered as an example of the former, inasmuch as adults, but not larvae, have been found living in fresh water (Knowlton and Williams, 1970). *Palaemonetes kadiakensis*, whose larvae cannot tolerate low concentrations of salts (Hubschman, 1975; Strenth, 1976), illustrates elimination of the need to breed in salt water—a manifestation of maximal specialization and adaptation to the fresh water environment persisting through the life cycle.

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THE COMPOSITION OF BLOOD PLASMA AND PARIETAL MUSCLE OF OSLO FJORD EELS [*ANGUILLA ANGUILLA* (L.)] AND THE RIVER LAMPREY [*LAMPETRA FLUVIATILIS* (L.)]

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Abstract—1. The principal osmotic constituents of eel and lamprey parietal muscle have been determined and summations of ions and nitrogenous constituents compared with those in the plasma.

2. By ultracentrifuging eel muscle, analysing the muscle fluid and centrifuged muscle, and determining their extracellular fluid (inulin space) ion-binding in the cells was estimated as Na 60%, K 4.6%, Ca 67%, Mg 28%, Cl 46% and P 1.3%.

3. Calculated osmolality of muscle exceeds that of the plasma, and this is discussed in relation to binding and other factors.

INTRODUCTION

The European eel *Anguilla anguilla* is well known as a teleost which spends part of its life in the sea and part in fresh waters. Most work on its salt and water balance has been done on specimens collected from fresh water, and studies have shown that when transferred to sea-water these show increases of about 15–20% in the osmotic concentration of the blood (Duval, 1925; Keys, 1932–1933; Boucher-Firly, 1935; Sharratt *et al.*, 1964). The major ions in parietal and tongue muscles of eels are correspondingly higher in those kept in sea-water for at least 2 weeks (Chan *et al.*, 1967).

Generally it has been found that the longer eels have been kept in sea-water (e.g. 10–12 days) the more the blood concentration comes back to near the values of those in fresh water. This is true of large yellow eels and especially of the mature silver eels—those that are ready to migrate down rivers on their spawning migration to the Sargasso Sea (Boucher-Firly, 1935). One of the large eels kept by Duval (1925) for 5 months in sea-water at the Monaco aquarium (1157 mOsm) when transferred to fresh water showed after 8 days a concentration of 361 mOsm, only slightly less than the 371 mOsm of those in the aquarium tanks.

The present study has been made on eels from sea-water in Oslo Fjord. These were immature "yellow" eels, not silver eels, trapped in the upper 1–5 metres of the Fjord. The work was carried out at the Biological Station of the University of Oslo, and at Glasgow, and is concerned with the inorganic ions, organic phosphates and nitrogenous compounds which are assumed to contribute to the total osmotic concentration of plasma and muscle. In the case of muscle it has involved the chemical determination of some 16 constituents.

A parallel study of the river lamprey *Lampetra fluviatilis* has been made since little information exists on the chemical composition of its muscle (Morris, 1972; Robertson, 1974).

MATERIALS AND METHODS

Eels captured in traps at a depth of 1–5 metres were obtained from the Drøbak Sound area of Oslo Fjord and kept at 12–16°C in gently running sea-water pumped from a depth of 50 metres. On several yearly visits during the months of July and August, this water showed only a slight variation in salinity, S 33–34‰ (18.7–19.2 g Cl/l), on the days the eels were used, whereas the surface waters at the station at the same time had values of S 16–25‰ (9–14 g Cl/l). Eels at times would be subject to higher salinities since they go deeper when surface temperatures are high, as was the case in the summer of 1982 when a maximum of 22–23°C was reached. The Director informs me that the monthly averages in salinity at 1 m depth during 1980–1982 ranged between S 17.00‰ and S 30.85‰, being highest during the winter months. The mean salinity at 1 m depth during the 3 years was S 24.4‰, equivalent to an osmolality of 718 mOsm and a chloride content of 13.71 g Cl/l.

The weight of the specimens was 90–120 g, the size 36–46 cm.

Lampreys taken from the river Severn in March were kept in Glasgow tap-water (from Loch Katrine) at 10°C. This is a soft water, with a composition at the time of 2.8 mg Ca/l and 7 mg Cl/l. The specimens were 28–38 cm in length and about 38–50 g, with gonads sexually differentiated.

Narcotization was affected by propylene phenoxytol, 60 drops shaken in 50 ml sea-water or fresh water per litre (Adams *et al.*, 1962), and then blood was withdrawn from the heart by pipette under liquid paraffin and placed in centrifuge tubes kept in a beaker of crushed ice. After centrifugation the green plasma, still under paraffin, was removed and used for analysis. Samples of white parietal muscle were taken from the trunk and tail region posterior to the anus (epaxial and hypoxial muscles), care being taken to remove the dermis completely. After gentle blotting with filter paper, samples were taken for trichloroacetic acid extraction (in proportions of *ca* 4 g in *Anguilla*, 2 g in *Lampetra* + 20 ml 10% TCA, the tissue being ground with quartz sand), zinc sulphate–barium hydroxide extraction (*ca* 4 or 2 g + 10 ml each of a 4% and 0.3 N solution), and for

dry weight (24 hr at 100–101 C), and ashing for cations. Ashing was carried out at 525–550 C in platinum basins in the presence of sulphuric acid, the ash being dissolved in 1–2 ml N/20 nitric acid before quantitative dilution for estimating the major cations by atomic absorption spectrophotometry. For calcium estimation lanthanium chloride was present in standards and samples at 1% final concentration of lanthanum ions to suppress interference from phosphate. The Aminco-Cotlove chloride titrator was used for some of the chloride measurements in TCA extracts.

Methods for phosphate fractionation and determination of nine nitrogenous constituents are those previously used (Robertson, 1980).The dipeptide carnosine found in large amounts in the Japanese and European eels (Konosu *et al.*, 1964; Huggins and Colley, 1971) was estimated by a diazotized sulphanilic acid method (Macpherson, 1946). Histidine reacts also with this carnosine reagent, so that its value, obtained by a pyridine-potassium tri-iodide method (Newman and Turnbull, 1960), was subtracted to get the carnosine content. Since the naphthoquinone-sulphonic acid α-amino acid reagent (Frame *et al.*, 1943; Russell, 1944) was found to react with carnosine to the extent of 1.16 times that of the glycine standard used, the appropriate correction was made so that the dipeptide value could be subtracted from the total α-amino-N, leaving the remainder as the "free amino acids" of Tables 1, 2 and 8.

Possible mutual interferences of nitrogenous constituents in the estimations were examined. In carnosine (0.05 mg) analysis there was no interference from creatine (0.04 mg) or betaine (5 mg). Ammonium-N (0.04 mg) gave a 4.6% increase, but at the concentration of NH_4^+ present in the eel filtrates analysed (0.01 mg NH_4-N per sample, taking a maximum value of 10 mg-ions/kg water), the effect is negligible. There were no significant effects on 0.02 mg creatine of adding 0.05 mg carnosine (+0.39%), 2.5 mg betaine (+0.86%), and only a slight effect of adding 0.02 mg NH_4-N (+1.4%).

Measurement of extracellular space were obtained by injecting 1.5–2 ml of a solution of 8% inulin in 33% seawater intraperitoneally and taking samples of blood and muscle after 17–25 hours. Inulin was estimated in zinc sulphate-barium hydroxide filtrates by measuring the fructose obtained after hydrolysis (Roe *et al.*, 1949), as also chloride, so that both inulin and chloride spaces were obtained from the same samples.

Samples of muscle after a short freezing were weighed and then ultracentrifuged at 20,000 rpm and RCF of 48,200 at 2–3 C for 1 hr. This short freezing was found necessary to get a reasonable amount of fluid, as otherwise only a small amount of mainly interstitial fluid was obtained. The supernatant and the residual (centrifuged) muscle were carefully collected and weighed. From 10 g muscle about 2 g fluid could be obtained. The eel plasma and muscle fluid were used as TCA and $Zn(OH)_2$ extracts for ion and inulin analyses, the centrifuged muscle usually ashed.

Considerable variation was found in the water content of eel muscles, correlated with their fat content. In the 26 specimens analysed the muscle water was 562–764 g/kg, mean and SD being 689 and 51. In a muscle with 58.6% water, the fat content was 18%. This was quite exceptional, since 21 of the 26 eels examined had muscle water contents within the 650–750 range. It was found that the amount of water could vary in different parts of the dorsal musculature, presumably in relation to the amount of fat. This causes difficulties in a study where concentrations of ions and nitrogenous compounds have to be given in terms of total water content. While most of the analyses can be done in one trichloroacetic acid filtrate, doing so led to the finding that the sodium values calculated from dilutions of the filtrate were higher than those from ashed material. This was traced to sodium liberated from the glass vials in which the extracts had been kept frozen or at 5 C. It is essential to keep all dilutions for atomic absorption analyses of sodium in plastic tubes, as well as using plastic bottles for the standards (1–5 mg Na/l). Filter paper (No. 1 Whatman) contaminates any dilute filtrates with extra sodium ions, but these seem to be easily washed out from the paper with distilled or deionized water. In tests of 3 week's duration of glass vials with a dilute solution of salts as chlorides approximating muscle ions in composition, slight release of ions from the glass seemed to occur from some of the tubes only, particularly sodium and magnesium, in both 10% TCA and distilled water.

A drawback in using dried muscle with a considerable fat content for analysis is the seeming impossibility of getting complete extraction of ions with TCA. Such muscle can be ashed for the cations, but for chloride fresh tissue has to be used.

RESULTS

Composition of plasma and whole muscle

From Table 1 it can be seen that Oslo Fjord eels kept 7–12 days in full-strength aquarium sea-water have plasma osmotic concentrations about 1/3 of that of the sea-water, with sodium and chloride forming 94% of the ions and magnesium little more than half the concentration of potassium and calcium. In four specimens kept in tap-water for 4–7 days, the osmolality of the plasma decreased by 9% with falls in all ions, but not in total phosphorus. All the falls are significant (t-test, $P = 0.05$) except for potassium.

The plasma of eels, coloured green by the presence of a protein–biliverdin (Oidé and Utida, 1967), has a relatively high concentration of protein (> 70 g/l as determined gravimetrically), which will have a negative charge as well as complexing with a proportion of the calcium. Cations in the plasma of the eels adapted to full-strength sea-water amount to 208.7 m-equiv/kg water. Adding lactate ion concentration of 1.89 mg-ions/kg water and bicarbonate ion concentration of 10.2 mg-ions/kg water to chloride and phosphate (assuming P as wholly inorganic with a valency of 1.8 at pH 7.4–7.5), the total anion concentration comes to 189.5 m-equiv/kg water leaving an anion deficiency of 19 m-equiv/kg water, which may be mainly proteinate anions, with a little sulphate. This deficiency will be slightly greater if some of the 10.8 mg-atoms/kg water P is associated with larger molecules; inoraganic P seems to be much less half the total concentration, and some phospholipid-P may be present.

In muscle the concentration of ions is 85–93% of that in the plasmas, with the usual high values of potassium and phosphate. These two ion concentrations and the relatively high magnesium values of 15 and 13 mg-ions are offset by the reductions of sodium and chloride to between the sixth and a seventh of their plasma values. Of the differences in ion concentrations between the two groups of eels (all concentrations were less in fresh water except sodium concentration) only magnesium and chloride ion differences were significant at the $P = 0.05$ level.

Nitrogenous constituents in the muscle were mostly free amino acids, the dipeptide carnosine, creatine and betaine, the last two having the highest concentrations. Trimethylamine oxide was absent and the urea concentration very small. The values in the table are those for the 14 analyses (including the eels in fresh water) in which carnosine was dis-

Table 1. Composition of blood plasma and parietal muscle of *Anguilla anguilla* in sea-water ($N = 22$) and fresh water ($N = 4$) (mean ± SE)

Constituent	mg-ions or mM per kg water				
	Sea-water	In sea-water		In fresh water	
		Plasma	Muscle	Plasma	Muscle
Sodium	463	189 ± 2.5	27.7 ± 1.5	172 ± 1.6	29.7 ± 2.1
Potassium	9.9	4.73 ± 0.14	131 ± 2.1	4.18 ± 0.19	126 ± 2.5
Ammonium	—	—	7.2 ± 0.7	—	4.6 ± 0.4
Calcium	10.1	4.93 ± 0.32	7.75 ± 0.33	3.01 ± 0.13	6.57 ± 0.22
Magnesium	52.8	2.55 ± 0.12	15.3 ± 0.35	1.70 ± 0.08	12.9 ± 0.52
Chloride	541 ± 1.1	158 ± 2.4	25.8 ± 1.3	138 ± 5.0	22.2 ± 1.1
Total P*	—	10.8 ± 0.73	110 ± 1.8	13.9 ± 0.86	107 ± 1.8
Total ions	1107†	370	325	333	309
Free amino acids	—	—	36.6 ± 3.5	—	23.9 ± 2.1
Carnosine	—	—	36.1 ± 1.7	—	28.8 ± 2.2
Creatine	—	—	57.9 ± 2.2	—	49.8 ± 3.5
Creatinine	—	—	1.1 ± 0.2	—	2.2
Betaine	—	—	51.8 ± 3.1	—	43.7 ± 4.3
Trimethylamine oxide	—	—	0.0	—	0.0
Urea	—	—	1.61 ± 0.29	—	0.81 ± 0.26
Total NPN*	—	—	399 ± 16	—	344 ± 24
Glucose	—	3.3 ± 0.8	4.0 ± 0.7	—	—
Protein g/l	—	73.5 ± 2.1	—	71.4 ± 3.6	—
Water g/l or g/kg	989	914 ± 2.7	698 ± 10.0	920 ± 5.8	640 ± 26.1
mOsm	1001	340 ± 5.2	—	310 ± 4.1	—

Chloride of fresh water = 5.45 mg/l (= 0.154 mg-ions/l).
NPN = non-protein nitrogen.
*mg-atoms/kg water.
†Including 27.9 mg-ions/kg water sulphate and 2.3 bicarbonate.

tinguished from the amino acids. Because of variation, the apparent fall in all these constituents when eels were put into fresh water was not statistically significant.

Plasma and muscle of *Lampetra fluviatilis* (Table 2) show broadly a similar pattern of ions and nitrogenous constituents to *Anguilla*, but at lower concentrations of all except plasma potassium. The ion concentrations of muscle were 88% of those in plasma, the very low calcium ion concentration of 1.40 mg-ions/kg water being perhaps correlated with the absence of calcified structures in the cyclostome.

Cation–anion balance of plasma, 150–140 m-equiv/kg water excludes ammonium and sulphate ions (0.4 and 5.4 in a previous analysis (Robertson, 1954)) leaving a probable deficit in inorganic anions which may be made up by proteinate. Protein concentrations are only half those in *Anguilla*.

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No obvious differences relating to sex were seen in any of the data from the three males and two females examined.

From Table 3 it can be seen that bound creatine in the three cases was rather low. It was measured from phosphate fractionation as creatine phosphate, whereas the total creatine was obtained by the α-naphthol-diacetyl method. In previous work on *Squalus* (Robertson, 1975), bound creatine came to about half of the total concentration. Both the amino acids arginine and proline were in low concentration.

Phosphate compounds in ice-cold TCA extracts of muscle were separated into 4 fractions: inorganic-P, creatine-P, ATP and a fourth fraction found by subtracting the sum of the first three from total P (Table 4). The inorganic fraction, which was usually the largest, was 40–45%; ATP, 4–7%; creatine-P, 4–6%; the remainder containing all other compounds, including any phospholipid-P. Compared to *Anguilla*. *Lampetra* showed markedly lower values of total phosphorus and the fourth fraction.

Intracellular ionic concentrations of Anguilla *muscle*

Extracellular fluid spaces in muscle were determined by inulin injections of eels in full-strength sea-water. On the assumption that lymph and interstitial fluid have the same ionic concentrations as plasma, values for this extracellular fluid were subtracted from whole muscle analyses, and the intracellular figures then recalculated per kg cell water.

Table 2. Composition of blood plasma and parietal muscle of *Lampetra fluviatilis* in fresh water ($N = 5$)

Constituent	mg-ions or mM per kg water	
	Plasma	Muscle
Sodium	138 ± 2.5	15.2 ± 0.72
Potassium	4.33 ± 0.41	90.6 ± 5.5
Ammonium	—	2.72 ± 0.33
Calcium	2.84 ± 0.60	1.40 ± 0.13
Magnesium	1.19 ± 0.12	8.37 ± 0.37
Chloride	121 ± 3.0	20.3 ± 0.85
Bicarbonate	4.0	—
Total P*	8.2 ± 1.0	77.7 ± 3.3
Total ions	280	216
Free amino acids	—	23.5 ± 3.9
Carnosine	—	8.4 ± 1.2
Creatine	—	42.5 ± 0.7
Creatinine	—	2.4 ± 0.7
Betaine	—	21.6 ± 7.9
Trimethylamine oxide	—	0.0
Urea	—	0.51 ± 0.4
Total NPN*	—	257 ± 9.6
Glucose	—	4.0 ± 0.2
Protein g/l	38.5 ± 2.8	—
Water g/l or g/kg	955 ± 4	686 ± 19.5
mOsm	254 ± 4.0	—

*mg-atoms/kg water.

Table 3. Creatine, arginine and proline of muscle (mM per kg water)

	Anguilla In sea-water ($N = 3$)	*Anguilla* In fresh water ($N = 4$)	*Lampetra* In fresh water ($N = 4$)
Creatine			
Free	46.6 ± 2.8	44.0 ± 2.4	37.7 ± 1.7
Bound	3.81 ± 0.82	6.38 ± 1.5	4.84 ± 1.1
Arginine	1.40 ± 0.12	1.26 ± 0.03	1.54 ± 0.21
Proline	0.84 ± 0.15	0.65 ± 0.08	0.39 ± 0.09

Table 4. Acid-soluble phosphate fractions in muscle (mg-ions (mM) per kg water)

	Inorganic P	Creatine P	ATP	Hexose P, etc.	Total P*
Anguilla					
In sea-water (3)	45.0 ± 0.9	3.81 ± 0.82	4.41 ± 1.24	35.0 ± 2.3	97.0 ± 2.0
In fresh water (4)	39.8 ± 3.3	6.38 ± 1.45	3.74 ± 0.58	42.8 ± 2.1	100.2 ± 1.9
Lampetra					
In fresh water (4)	31.7 ± 0.9	4.84 ± 1.12	4.42 ± 0.11	20.4 ± 2.0	70.2 ± 1.6

*Includes 3 P-atoms of ATP.

The mean values from 13 experiments are given in Table 5. All ion concentrations were slightly higher in the cells than in whole muscle, except for sodium and chloride since these were greatly reduced owing to the subtraction of the sodium and chloride-rich extracellular fluid. Muscle cell K/Na ratio was now 19 and the cell/plasma potassium ratio 32. The sodium and chloride ion concentrations of the cells were only 1/24 and 1/15 respectively of their concentrations in the plasma.

This intracellular composition in eel muscle was in general agreement with the pattern found in the muscles of other animals, except perhaps for the calcium. Its calculated higher value in the cells than in the plasma, ratio 1.66, may be an over-estimate due to the presence in the muscle of small fragments of the intermuscular bones, as explained below.

Osmolality of plasma and muscle

An attempt has been made to convert the analytical data on *Anguilla* and *Lampetra* to milliosmoles by the use of osmotic coefficients. Since sodium and chloride constituted 93–94% of the ions in plasma, it seems reasonable to use the coefficients of a NaCl solution of the same molality as the plasma, that is, those for 0.34, 0.31 and 0.254 M, for the respective ionic concentrations. These coefficients are 0.921, 0.921 and 0.922 (Robinson and Stokes, 1965). But the complexity of muscle composition and the possibility of ion binding to large molecules present serious difficulties calculating osmotic coefficients for the muscle constituents.

Taking the simplified view that the ions and organic compounds are freely in solution in muscle water, an approximate conversion to osmolality can be made. The major muscle ions of *Anguilla* in sea-water can be considered to be a solution of the following salts: 27.7 mM NaH_2PO_4; 33.74 mM KH_2PO_4; 48.56 mM K_2HPO_4; 7.75 mM $CaCl_2$; and 15.3 mg-ions(Mm)/kg water $MgCl_2$. The ionic composition of a solution of these salts is identical with the values in Table 1, except for Cl^-, which is 46.1 mg-ions/kg water instead of 25.8. From the osmotic coefficients of these salts (Robinson and Stokes, 1965) at a molality of 0.34 M, it can be calculated that this hypothetical muscle salt solution would have an osmolality of 272.58 mOsm. Since the analytical composition is 337.71 mg-ions(Mm)/kg water, the osmotic coefficient is 272.58/337.71 = 0.807.

An attempt to verify the correctness of this value failed when the final addition of $CaCl_2$ to a solution of the other salts brought about precipitation of calcium phosphate. Omitting the $CaCl_2$, a solution of the other muscle salts gave a measured osmolality of 256 mOsm instead of the theoretical 252 (+1.6%), a coefficient of 0.83. A value of 0.81 has been taken for the two *Anguilla* analyses, and 0.82, another calculated value, for *Lampetra* ions.

For the organic constituents a value of 1 has been taken except for the values of betaine, which can be calculated to be 1.05 at 0.34 and 0.31 M, 1.04 at 0.254 M (Smith and Smith, 1940). The osmolality of a solution containing nitrogenous constituents in the concentrations found in the muscle of sea-water *Anguilla* specimens (glycine, 36.6 mM, representing the amino acids; carnosine, 36.1 mM; creatine, 57.9 mM; betaine, 51.8 mM; a total of 182.4 mM) was measured as 178 mOsm. At this molality (0.1824 M), using osmotic coefficients of 0.983 for glycine (Smith and Smith, 1937), 1.03 for betaine and 1 for carnosine and creatine, the calculated osmolality is 183 mOsm. The small discrepancy would

Table 5. Intracellular composition of *Anguilla* muscle (specimens from sea-water, $N = 13$)

	mg-ions (mM) per kg water						g/kg or g/l
	Na	K	Ca	Mg	Cl	P*	H_2O
Muscle	27.9 ± 2.3	133 ± 3.2	7.8 ± 0.5	15.9 ± 0.3	26.8 ± 2.0	109 ± 2.5	713 ± 8.6
Muscle cells	7.82 ± 2.33	149 ± 3.2	8.2 ± 0.5	17.5 ± 0.4	10.5 ± 1.6	121 ± 2.5	691 ± 10.1
Plasma	192 ± 2.6	4.67 ± 0.18	4.95 ± 0.21	2.69 ± 0.11	157 ± 3.2	10.2 ± 0.95	915 ± 3.3
Ratio: muscle cell/plasma	0.041	31.9	1.66	6.51	0.067	11.9	0.76

Inulin spaces as % muscle water: 11.12 ± 0.67.
Chloride spaces as % muscle water: 16.98 ± 1.00.
*mg-atoms/kg water.

be less if the purity of the chemicals was only 98–99% and if the osmotic coefficients of carnosine and creatine were actually slightly less than 1.

With this justification for the particular osmotic coefficients used, the analyses of Tables 1 and 2 have been converted to milliosmoles, as shown in Table 6. The calculated and determined values of the plasmas agree well, but the calculated osmotic concentration of the muscles exceeds that measured in the plasmas by 27–33% in *Anguilla* and 8.7% in *Lampetra*. In explanation of these discrepancies, there are several possibilities: binding of some of the ions and nitrogenous constituents to larger molecules, especially proteins, so that their analytical values do not represent their true osmotic concentrations; some breakdown of labile complexes from their *in vivo* states (e.g. creatine phosphate splitting into two molecules); NH_4^+ ions released from some compounds; osmotic coefficients of the organic phosphates different from those of the inorganic phosphates; phospholipid P (mean value in *Anguilla* muscle 6.1 mg-atoms/kg water according to Zwingelstein *et al.*, 1975) not ionic; and analytical error. Some minor errors must exist since in *Anguilla* muscle the N-atoms from the known constituents, including NH_4^+ exceed the total NPN of the micro-Kjeldahl analysis by 11 and 5.8%, despite careful checking of mutual interferences in the estimation of the various nitrogenous compounds to avoid positive errors.

Possible binding was investigated. By ultracentrifuging muscle and comparing analyses of the fluid which separates as supernatant above the centrifuged muscle with analyses of the centrifuged muscle, an indication of binding or sequestration of ions can be obtained. This muscle fluid will contain some extracellular fluid, not necessarily in the same proportion per kg solvent water as in the original muscle. These extracellular spaces were measured in five eels into which inulin had previously been injected. By (1) weighing the original samples of the muscles and then the final weights of fluid and centrifuged muscle, and (2) measuring the inulin spaces in the fluid and centrifuged muscle, and water and ionic composition of these two components, the data of Tables 7 and 8 were obtained. To avoid the errors of using separate muscle samples with their possible variations in ions and in fat, consequently water, the values for whole muscle were calculated from its components (fluid and centrifuged muscle).

Table 7 shows that centrifuged muscle has a lower water content than whole muscle owing to the loss of muscle fluid. However, on a water content basis its K^+, Ca^{2+} and Mg^{2+} are higher, only Ca^{2+} and Mg^{2+} significantly so (t-test), with Na^+ and Cl^- about the same. Muscle fluid has significantly less K^+, Ca^{2+} and Mg^{2+} than the centrifuged muscle, but the lower P (PO_4^-, PO_4^{2-}) is not statistically significant. These differences arise in part from different amounts of extracellular fluid, and in part from binding and/or sequestration of some of the ions. It should be mentioned that the calcium determinations in whole muscle may be too high, since it proved impossible to identify and pick out completely, from either fresh or dried centrifuged muscle, pieces of the intramuscular bones (epineurals and epipleurals, Tesch, 1977) which would contain some phosphate and carbonate salts. The lowest of the Ca^{2+} values in five or six samples of each centrifuged muscle were taken for the Table.

The concentrations of ions in the muscle cells of the five eels are given in Table 8. Compared with whole muscle, Na^+ and Cl^- ion concentrations are much reduced owing to the subtraction of the Na-rich and Cl-rich extracellular fluid, but the other ions are higher, as already shown in Table 5, based on these and another eight analyses. Values for the cellular portion of muscle fluid, obtained by subtracting the ions of its extracellular component (inulin space) and recalculating per kilogram cell water, are lower than those of the muscle cells, all significant at the $P = 0.05$ level (t-test) except K^+ and P. On a percentage basis the binding or sequestration is least with K^+ and greatest with Ca^{2+}, Na^+, Cl^- and Mg^{2+}. Although some of the reduction may be due to sequestration of ions in cell organelles, most may be due to binding to muscle proteins.

Table 6. Calculated osmolality: mOsm per kg water

	Anguilla		*Anguilla*		*Lampetra*	
In sea-water	In fresh water		In fresh water			
	Plasma	Muscle	Plasma	Muscle	Plasma	Muscle
Inorganic ions and phosphates	341	263	307	250	258	177
Free amino acids	—	36.6	—	23.9	—	23.5
Carnosine	—	36.1	—	28.8	—	8.4
Creatine and creatinine	—	55.2	—	45.6	—	40.1
Betaine	—	54.4	—	45.4	—	22.5
Urea	—	1.6	—	0.8	—	0.5
Glucose	3.3	4.0	—	—	—	4.0
Total determined constituents	344	451	307	395	258	276
NPN unaccounted for*	—	Nil	—	Nil	—	20
Osmolality as determined	340	—	310	—	254	—

Osmotic coefficients used: *Anguilla*—plasma ions 0.921, muscle ions 0.81, betaine 1.05, 1.04, and 1.0 for other organic constituents; *Lampetra*—plasma ions 0.922, muscle ions 0.82, betaine 1.04, 1.0 for other constituents. Bound creatine subtracted from total creatine as it is included with the phosphates.

*mg-atoms/kg water.

Table 7. Ionic composition of plasma and muscle components ($N = 5$)

	mg-ions (mM) per kg water						
	Na	K	Ca	Mg	Cl	p*	g/kg H_2O
Plasma	199.6 ± 1.65	4.72 ± 0.22	5.62 ± 0.05	2.42 ± 0.08	150.0 ± 2.12	7.46 ± 0.41	907† ± 3.4
Whole muscle	23.6 ± 0.47	140.7 ± 1.49	8.94 ± 0.40	16.0 ± 0.28	21.5 ± 0.44	115.5 ± 3.46	718 ± 19.6
Centrifuged muscle	23.5 ± 0.90	144.7 ± 2.48	11.01 ± 0.68	17.7 ± 0.46	21.60 ± 0.46	117.3 ± 3.97	678 ± 26.2
Muscle fluid	23.9 ± 0.86	130.3 ± 1.89	3.33 ± 0.08	11.4 ± 0.21	21.28 ± 0.56	110.9 ± 2.52	856 ± 2.2

Osmolality of plasma in above specimens 341.1 ± 2.89 mOsm, sea-water value 1008 mOsm.
*mg-atoms/kg water.
† = 921 ± 6.2 g/l.

Table 8. Intracellular composition of muscle, and approximate amount of ion binding or ion sequestration

	mg-ions (mM) per kg water					
	Na	K	Ca	Mg	Cl	p*
Muscle cells	7.23 ± 0.59	153.4 ± 2.34	9.26 ± 0.46	17.29 ± 0.37	9.56 ± 0.87	125.5 ± 3.64
Cellular portion of muscle fluid	2.85 ± 1.02	146.4 ± 2.20	3.03 ± 0.09	12.50 ± 0.31	5.14 ± 1.31	123.9 ± 2.78
Difference = bound or sequestered ions	4.37	7.0	6.23	4.79	4.42	1.6
Bound or sequestered ions %	60	4.6	67	28	46	1.3

Data based on inulin spaces of 8.51 ± 0.53%, whole muscle; fluid centrifuged from muscle, 11.35 ± 0.78%; muscle after centrifugation, 7.46 ± 0.43% ($N = 5$).
*mg-atoms/kg water.

The apparent large binding or sequestration of Na^+ and Cl^- is in the muscle cells. In whole muscle most of these ions are in the extracellular component (81 and 79% respectively). A minor point is that not all the ions in the cellular portion of the muscle fluid may be wholly free. Since centrifuged muscle fluid itself has a high protein content (84.6 g/l in one specimen compared to the 71–74 g/l of the plasma in the specimens in Table 1), it is probable that some of the Ca^{2+} ions are bound in a calcium–protein complex.

Applying this binding data to the ions in the muscle of sea-water adapted eels (Table 1) after subtracting those in the extracellular space (11.12% of muscle water), one finds 27.2 bound ions out of a total 311 intracellular ions (excluding NH_4^+), that is 8.75%. In whole muscle there is a reduction from the total of 325 (Table 1) to 300.5 free and 24.2 bound ions, leading to a fall in calculated osmolality (Table 6) from 451 to 431 mOsm, reducing the 33% overestimate to only 27%.

It is improbable that eel muscle is strongly hyperosmotic to extracellular fluid and plasma.

The breakdown of labile compounds or loosely bound nitrogenous compounds has not been investigated except for creatine phosphate. It may be that some complexes are broken down by TCA extraction and perhaps not all the amino acids are free. ATP and creatine phosphate break down easily and despite ice-cold conditions and rapid neutralization of the TCA extracts, the values of these constituents in Table 4 may be too low. If one accepts the opinion of Watts and Watts (1974) that in the resting fish creatine is almost wholly phosphorylated, this would lead to a reduction of estimated osmolality of muscle from 451 mOsm (Table 6) to near 397 mOsm, still 16.8% greater than the plasma value, but allowing for estimated ion binding the reduction would be to 377 mOsm, which is 10.9% above the plasma concentration.

It is concluded that a considerable proportion of the inorganic and organic constituents found by analysis must be bound as complexes with the muscle proteins *in vivo*, not exerting the osmotic effect calculated from their concentrations. Accepting at face value the analyses and calculations in *Anguilla* and *Lampetra*, it would seem that such binding is much less in the latter.

DISCUSSION

The osmotic concentration of 14 yellow eels from sea water of 1000 mOsm examined at Drøbak ranged between 320 and 358 mOsm, with a mean of 340 ± 5.2 (SE). Other data for *Anguilla anguilla* captured in the sea are 341 mOsm, the mean of two groups of 333 and 349 mOsm (numbers and physiological state not given) from sea-water of 1028 mOsm off Helgoland (Dakin, 1908), and 374 and 356 mOsm from Drøbak and Trondheim (Schmidt-Nielsen and Schmidt-Nielsen, 1923). Whether the latter were immature (yellow eels) was not stated. However, the present mean value of 340 mOsm is in line with a fairly complete analysis of the plasma, adjusted by a reasonable osmotic coefficient to give an estimated osmolality of 344 mOsm.

The analytical values for plasma in Table 1 agree fairly well with data on yellow eels trapped in brackish water at the estuary of the river Nordre Älv, near Göteborg, and kept for at least 8 days in water of salinity S 25–27‰ (Larsson and Fänge, 1969), equivalent to about 740–800 mOsm. The mean values from 6–26 individuals compared to those of Table 1, converted to a litre basis are (J.D.R.'s in brackets): Na, 165 (173); K, 4.5 (4.3); Ca, 7.4 (9.0); and Cl, 144 (144) m-equiv/l. The principal differences are the lower Na (−4.6%) and Ca (−17.8%) of the Göteborg analysis. Some Göteborg yellow eels kept in fresh water for 2–3 weeks gave mean values of Na 149 and Cl 112 m-equiv/l. Corresponding values from Table 1 are 158 and 127 respectively.

Eels from sea-water transferred to fresh water show much smaller changes in osmotic concentration than fresh water eels transferred to sea-water. The Drøbak eels showed only a 9% fall after 4–7 days in fresh water, mean values being 340 and 310 mOsm. Dakin (1908) gives 3 values 312–315 mOsm of marine eels transferred to fresh water at Kiel, a fall of 8% from values of eels caught off Helgoland (341 mOsm). A relative insensitivity to change was found also by Duval (1925) at Monaco, where eels in aquarium sea-water of 1157 mOsm had concentrations of 350–371 mOsm after 5 months, whereas one of the same group of large (silver?) eels had a concentration of 361 mOsm after 8 days in fresh water. This may be contrasted with values of 328 mOsm in fresh water and 393 mOsm after 4–10 days in sea-water of 1023 mOsm in eels (phase unstated) studied by Keys (1932–33), and 326 and 374 mOsm in mature silver eels (Sharratt *et al.*, 1964).

Comparative data on the composition of muscle in fresh water yellow eels are only broadly in agreement. Total cation concentrations vary between 159 and 175 mg-ions(mM)/kg water (McCance, 1944) and 189 (Chan *et al.*, 1967) compared with those of 175 in Table 1, with the usual high values of potassium 90–126. The major differences are the high sodium values 38–70 mg-ions/kg water in McCance's eels, 27–30 in the other analyses, and high magnesium of 29 mg-ions/kg water in the values of Chan *et al.*, compared to 10–13 in the others. The gravimetric analysis of Katz (1896) of muscle of two large eels (fat content of muscle 21.8%, yellow or silver?) agrees fairly well with the analyses of McCance (except for sodium of 22 mg-ions/kg water) and that in Table 1.

In yellow eels equilibrated for 4–5 weeks in sea-water, the concentration of muscle cations comes to 209 mg-ions/kg water (Chan *et al.*, 1967) as against 182 mg-ions/kg water of Table 1. The concentrations of sodium and magnesium are 43 and 30 mg-ions/kg water respectively compared with 28 and 15 mg-ions/kg water. The magnesium values of Chan *et al.* are probably much too high, as the Mg/Cl molar ratio calculated from their Scarborough sea-water of 0.1333 is 36.4% higher than the standard figure of 0.0977 obtained from Barnes's (1954) tables.

Three ionic concentrations of muscle cells of the American eel *Anguilla rostrata*, obtained by using an extracellular space of 10% determined by ^{14}C-labelled polyethylene glycol using a distribution time of 24 hr

(Schmidt-Nielsen *et al.*, 1972), may be compared with those of *A. anguilla*. The four American eels had been kept at least 2 weeks in flowing sea-water of 934 mOsm. Expressed as mg-ions/kg of cell water (mM), these means were (*A. anguilla* in brackets): Na, 12.41 (7.83); K, 147.8 (149); and Cl, 9.67 (10.5). But plasma sodium and chloride of the American eel were only 83 and 86% of these ions in the Oslo Fjord eels.

In *Lampetra* osmotic and ionic concentrations in plasma and muscle are much lower than in *Anguilla* in fresh water, except for plasma potassium. Values for plasma fall within the range of *L. fluviatilis* as collected by Robertson (1974) and the analysis of Logan *et al.* (1980). The latter found a total concentration of 248.7 ± 9.5 (14) compared with the 254 ± 4.0 (5) of Table 2, a 2% difference in specimens from the same river (Severn); the differences will be slightly less osmotically as the first figure is an osmolarity, the second figure an osmolality. Their sodium and chloride figures are, however, below those in Table 2 by about 6–7%, even if allowance is made for the difference in units, on a litre and on a kg water basis.

Regarding the nitrogenous constituents of eel and lamprey muscles the same broad pattern exists, creatine and betaine in large amounts, lesser amounts of free amino acids and carnosine, negligible urea (<2 mM), and no trimethylamine oxide. The only significant differences in these compounds between lampreys and the fresh-water-adapted eels is the much lower carnosine and betaine of the former. Betaine has previously been isolated from lamprey muscle in amounts equivalent to 6.6 mM/kg water content (Strack *et al.*, 1937).

The absence of trimethylamine oxide and the large amounts of carnosine seem to be characteristic of the Anguillidae. Most marine teleosts have TMAO in their muscles, and one might have expected at least a slight accumulation of exogenous origin. The euryhaline flounder *Platichthys flesus* has 30.3 mM per kg when taken from sea-water and 20.0 mM when in fresh water (Lange and Fugelli, 1965).

Muscle carnosine of 40.9 mM/kg water was found in *A. japonica* reared in fresh water ponds (Konosu *et al.*, 1964). This compares with the 29–36 mM in Table 1, but much lower figures for *A. anguilla* (silver eels) are given by Huggins and Colley (1971), 13.7 mM when in fresh water, 14.4 when in sea-water. Free amino acids amounting to 14.4 and 23.4 mM were found in these eels, compared to 24 and 37 mM of the present investigation. *A. japonica* had 15.5 mM amino acids, so that free amino acids and carnosine come to a total of 56.4 mM, compared to the total of 52.7 mM of the Oslo Fjord eels in fresh water.

The binding of ions in eel muscle was investigated by comparing concentrations in the original cells and in the cellular component of the muscle fluid obtained by ultracentrifugation. For each ion, binding and/or sequestration amounted to: Na, 60%; K, 4.6%; Ca, 67%; Mg, 28%; Cl, 46% and P, 1.3% (Table 8). Since the smallest percentages were in the two highest concentrations, K and P, the amount of binding appeared to be no more than 9% of the total intracellular ions. This left a calculated over-estimate in whole muscle osmolality of 27%. Since the osmotic coefficients used in calculating the osmolality seemed to be supported by osmotic concentration measurements on solutions containing the salts and main organic constituents of muscle, it must be concluded that *in vivo* binding of ions and of some of the nitrogenous constituents to macromolecules must be considerable.

Other techniques have been used to estimate bound ions. Measurements of intracellular activity by means of ion-selective microelectrodes and analysis of intracellular concentrations have shown that in skeletal muscle there is some degree of binding to protein and/or compartmentation of chloride, sodium and potassium (Walker and Brown, 1977). There is still uncertainty about the amount of bound or osmotically inactive water. In single fibres of the barnacle *Balanus nubilus*, it is claimed that the solvent water is only about 68% of the fibre water, about 6.5% being extracellular in the cleft-tubular system, and the remaining 25–26% osmotically inactive. Calculations from microelectrode studies on this barnacle show apparent binding of 73% of the sodium, 13% of the potassium and 32% of the chloride (Hinke, 1970; Hinke *et al.*, 1973). In the decapod crustacean *Callinectes* a bound water of 29–36% has been calculated from electrophysiological, osmometric and ion analyses studies on single muscle fibres (Hays *et al.*, 1968).

From purely analytical considerations, it seemed unnecessary for the author to postulate any significant amount of bound water in *Nephrops* (Norway lobster) muscle, since the concentrations of ions and nitrogenous constituents expressed in terms of total water, and allowing for ion-binding, came to 92% of the plasma concentration and 90% of the concentration of the muscle press juice (Robertson, 1961).

In the marine fishes *Squalus* and *Chimaera*, and in the marine cyclostome *Myxine*, it also appears that on a total water content basis the principal soluble muscle constituents come at least to 103%, 94% and 95% respectively of the osmotic concentration of the plasmas, and these calculated values would be increased if some of the remaining non-protein-nitrogen could be allocated to other nitrogenous compounds (Robertson, 1975; 1976).

A non-solvent space anything like 25–36% of total water would lead to a large over-estimate of osmotic concentration of the muscles in these species and a much larger calculated over-estimate in the eels studied in this paper. It seems very unlikely that there is any large fraction of non-solvent or bound water in fish muscle.

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BIMODAL RESPIRATION OF INTERTIDAL PULMONATES

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Abstract—1. Aerial and acquatic rates of oxygen consumption were determined for the intertidal pulmonates *Benhamina obliquata* and *Siphonaria zelandica* at 10°C.

2. There was no significant difference between the levels of oxygen consumption of *Benhamina obliquata* in air and water but the aerobic metabolism of *Siphonaria zelandica* was significantly reduced during submersion.

3. Rates of oxygen consumption of the rocky shore pulmonates are similar to those of intertidal prosobranchs in aquatic and aerial environments.

4. The ratios of aquatic to aerial oxygen consumption for pulmonates from mangrove, salt marsh, mud flat and rocky shore habitats are correlated with the presence or absence of a secondary gill.

INTRODUCTION

The relative ability of pulmonates to respire aerobically in aerial and aquatic environments has been correlated with their principal organs of gas exchange (McMahon and Russell-Hunter, 1975, 1977, 1981; Houlihan, 1979). The oxygen consumption of pulmonates such as the high salt marsh snail *Melampus bidentatus*, which has a well developed lung and no vestige of a a ctenidium, is greatly reduced during periods of submersion (McMahon and Russell-Hunter, 1975, 1981). In contrast, pulmonates which have evolved a secondary gill, e.g. the high mud flat snail *Amphibola crenata*, can maintain aerobic metabolism during submersion (Shumway, 1981; Shumway and Marsden, 1982).

In the present study the patterns of aerial and aquatic respiration of two intertidal rocky shore pulmonates, *Benhamina obliquata* and *Siphonaria zelandica*, are investigated. Both species belong to the family Siphonariidae and possess a well developed lung and secondary gill (Cotrell, 1910; Purchon, 1968; Fretter and Peake, 1975).

The two rocky shore siphonariids are characterized by their limpet-like form and relatively high distribution in the intertidal zone. *Benhamina* is a notable member of the New Zealand fauna and is distributed from Cook Strait southwards. It is conspicuous by its size and is the largest high shore mollusc found in New Zealand, growing to a length of 65 mm and dry weight in excess of 1 g (Powell, 1979).

The ratios of aquatic to aerial oxygen consumption of thse two intertidal snails will be compared with those of other pulmonates with reference to the principal respiratory organs of each species. The patterns of oxygen consumption of the rocky shore pulmonates will also provide an interesting contrast with those described for a number of gill-bearing intertidal prosobranchs (Micallef, 1967; Micallef and Bannister, 1967; Hughes, 1971; McMahon and Russell-Hunter, 1977; Branch and Newell, 1978; Branch, 1979; Houlihan, 1979; Houlihan and Innes, 1982a; Innes, 1982).

MATERIALS AND METHODS

This work was carried out at the Portobello Marine Laboratory, New Zealand between August and October 1982. The pulmonates under investigation were *Benhamina obliquata* (Sowerby, 1825) and *Siphonaria zelandica* (Quoy and Gaimard, 1833). Both species are distributed between the mid and upper tidal levels of rocky shores (Morton and Miller, 1968; Powell, 1979). Experimental animals were collected from Allens Beach, Portobello.

Immediately after collection the animals were placed in a running seawater aquarium and held, without access to food, for 5–8 days prior to experimentation. The temperature of the seawater in the aquarium varied between 7 and 10°C and the salinity was relatively constant at 33.5‰. All experiments were carried out at 10°C and animals were held at the experimental temperature for 2 hr prior to respirometry commencing. Experiments took place in constant temperature water baths controlled to ±0.1°C.

Aerial rates of oxygen consumption of individual animals were measured using respirometers described by Davies (1966a). The snails were allowed to recover mantle cavity fluid prior to experimentation (Houlihan *et al.*, 1981) and aerial respirometry was carried out as described in Houlihan and Innes (1982a) using 20% solution of potassium hydroxide to absorb carbon dioxide. Micrometer adjustments were made every 10 min. The position of the snail in the respirometer was monitored and rates of oxygen consumption were calculated over a period when the animal was seen to be inactive. All rates of oxygen consumption have been converted to S.T.P.

Aquatic oxygen consumption was determined using a closed bottle technique as described by Houlihan (1979). The snails were allowed to attach and settle inside the respirometer bottles in running aerated seawater 1 hr before the flasks were sealed. The seawater in the bottles was stirred every 5 min with a magnetic stirrer and the experiment was terminated for any animals seen to be active. A blank bottle without an animal was run as a control with each set of experiments. The oxygen content of the seawater at the beginning and end of each experiment was determined by the Winkler technique (Strickland and Parsons, 1972). From the decline in oxygen content of the seawater (after adjustments for control), the volume of water in the flask and the duration

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of the experiment, the aquatic oxygen consumption of inactive animals was calculated. The oxygen content of the seawater was never allowed to fall below 80% of the saturated value during experiments (Innes, 1982).

At the end of each experiment the snails were removed from their shells and dried to a constant weight at 65°C. A wide size range of animals was used to determine relationships between dry weight and oxygen consumption. The mean dry weight ±SE of all the *Benhamina obliquata* used in experiments is 393.8 ± 46.2 mg ($N = 39$) whilst that of the smaller *Siphonaria zelandica* is 59.9 ± 4.5 mg ($N = 45$). Comparisons between aquatic and aerial rates of oxygen consumption are made using standard dry flesh weight animals of 400 mg for *Benhamina obliquata* and 60 mg for *Siphonaria zelandica*.

All results were subjected to regression and covariance analysis (Snedecor and Cochran, 1972) following the procedure of Wilson (1975). The results of regression analyses using data transformed by logarithms to the base 10 are shown in their logarithmic form. The criterion of significance is a probability of 5% or better. Means ±SE are used throughout.

RESULTS

Benhamina obliquata

Aquatic and aerial rates of oxygen consumption of *Benhamina obliquata* are plotted as a function of dry flesh weight in Fig. 1.

The relationships between dry flesh weight (X, mg) and oxygen consumption (Y, $\mu l O_2\ hr^{-1}$) are described by the regression analyses:

Air $\log_{10} Y = 0.709 \log_{10} X + 0.234$, where $N = 19$ and $r = 0.95$.

Seawater $\log_{10} Y = 0.627 \log_{10} X + 0.383$, where $N = 20$ and $r = 90$.

The correlation coefficients determined for the aerial and aquatic regression analyses are both significant ($P < 0.05$). Analysis of covariance revealed no significant differences in either slope or elevation between the respiration data obtained in air and seawater. The ratio of aquatic to aerial oxygen consumption for a standard weight (400 mg) animal is 1 : 1.16.

Siphonaria zelandica

Rates of oxygen consumption in air and water of a size range of *Siphonaria zelandica* are shown in Fig. 2.

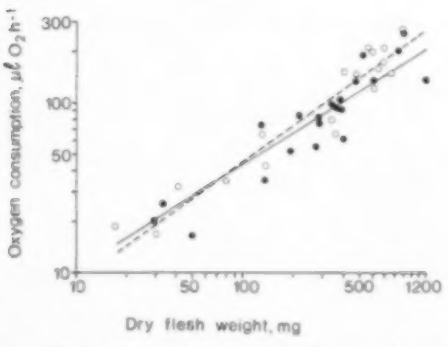

Fig. 1. Relationships between dry flesh weight (mg) and oxygen consumption ($\mu l\ O_2\ hr^{-1}$) at 10°C in air (○---○) and seawater (●—●) for *Benhamina obliquata*; the lines are drawn from the regression analyses shown in the text.

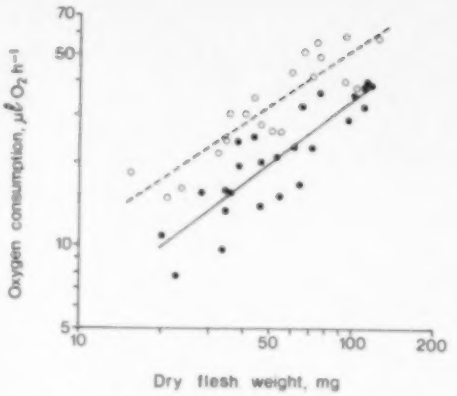

Fig. 2. Relationships between dry flesh weight (mg) and oxygen consumption ($\mu l\ O_2\ hr^{-1}$) at 10°C in air (○---○) and seawater (●—●) for *Siphonaria zelandica*; the lines are drawn from the regression analyses shown in the text.

The regression analyses describing the relationships between dry flesh weight (X, mg) and oxygen consumption (Y, $\mu l\ O_2\ hr^{-1}$) are:

Air $\log_{10} Y = 0.659 \log_{10} X + 0.385$, where $N = 20$ and $r = 0.89$

Seawater $\log_{10} Y = 0.772 \log_{10} X - 0.020$, where $N = 25$ and $r = 0.87$.

The relationships between dry weight and oxygen consumption in air and water are both significant ($P < 0.05$). Analysis of covariance showed that the aerial rate of oxygen consumption of *Siphonaria zelandica* is significantly higher ($P < 0.001$) that the aquatic rate. There was no significant difference in the weight exponents between aquatic and aerial environments. The aquatic to aerial ratio for a standard weight (60 mg) snail is 1 : 1.60.

DISCUSSION

The intertidal rocky shore pulmonates *Benhamina obliquata* and *Siphonaria zelandica* are able to respire aerobically following submersion. In this respect, *Benhamina obliquata* is similar to the mud flat snail *Amphibola crenata* which can maintain the same rate of oxygen consumption in air and water (Shumway, 1981; Shumway and Marsden, 1982). During submersion, the respiration rate of *Siphonaria zelandica* was significantly reduced by up to 40%. In aerial conditions, both pulmonates respire atmospherically with the pneumostome open and part of the mantle cavity functioning as a lung. Recent ultrastructure and respiratory studies by Wong (1980) on *Siphonaria zelandica* suggest that, in addition to pulmonary respiration, cutaneous gas exchange also occurs. Oxygen consumption measured following closure of the pneumostome suggests that up to 25% of the total aerial gaseous exchange occurs in this way, probably across the sides of the foot. This contrasts greatly with freshwater pulmonates where this type of cutaneous respiration is frequently the chief means of respiratory exchange. Alberts (1966), for example, demonstrated that in *Bulinus africanus*, *Bulinus tropicus* and *Lymnaea natalensis* cutaneous respiration may be from half to

Table 1. Ratios of aquatic to aerial oxygen consumption for a number of lung-bearing gastropods

Species	Habitat	Temperature (°C)	Aquatic to aerial ratio	Reference
Pulmonata:				
Melampus bidentatus	High tidal salt marsh	10–20	1:3.0–5.0	McMahon and Russell-Hunter (1981)
Cassidula aurisfelis	High tidal mangrove vegetation	28	1:6.01	Houlihan (1979)
Amphibola crenata†	High tidal mud flats	15	1:0.94*	Shumway (1981)
Benhamina obliquata†	Intertidal rocky shore	10	1:1.16*	Present study
Siphonaria zelandica†	Intertidal rocky shore	10	1:1.60	Present study
Prosobranchia:				
Marisa cornuarietis†	Freshwater	20	1:0.93*	Freiburg and Hazlewood (1977)
Pomacea paludosa†	Freshwater	20	1:0.91*	Freiburg and Hazlewood (1977)
Pomacea lineata†	Freshwater	25	1:1*	dos Santos and Mendes (1981)

Ratios have been calculated at the temperatures indicated for standard weight animals from information supplied in the references; the habitat of each species is indicated; *, denotes no significant difference between aquatic and aerial oxygen consumption; †, indicates those species which possess a gill.

eight times the pulmonary value. In the marine pulmonates studied so far, it appears that the ability to maintain the rate of aquatic respiration during submersion is associated with the development of a gill which is evidently secondarily developed after the loss of the original ctenidium.

As *Benhamina* is commonly distributed higher in the intertidal than *Siphonaria* (Borland, 1950; Morton and Miller, 1968), one may have expected *Siphonaria* to have evolved a more efficient system of oxygen uptake in seawater. This would not appear to be the case in view of the rates of aquatic oxygen consumption recorded for *Siphonaria* (Fig. 2) in the present study.

The weight coefficients reported for the siphonariid limpets in the present investigation (Figs 1 and 2) are higher than those reported for *Amphibola crenata* (Shumway, 1981; Shumway and Marsden, 1982) but similar to those described for a number of other pulmonates (von Brand *et al.*, 1948; Berg and Ockelmann, 1959) and prosobranchs (Davies, 1966b; Innes and Houlihan, 1981; dos Santos and Mendes, 1981; Innes, 1982). In common with a number of prosobranchs (Hughes, 1971; Houlihan, 1979; Houlihan and Innes, 1982a) the weight exponents of the intertidal pulmonates did not change significantly between aquatic and aerial environments (Figs 1 and 2).

The ratios of aquatic to aerial oxygen consumption of the amphiboliid (Shumway, 1981) and siphonariid (present study) pulmonates contrast with those of the salt marsh pulmonate *Melampus bidentatus* (McMahon and Russell-Hunter, 1975, 1981) and the tropical mangrove snail *Cassidula aurisfelis* (Houlihan, 1979) whose greatly depressed rates of aerobic metabolism during submersion (Table 1) may be correlated with the absence of a secondary gill.

A comparison is also made in Table 1 between the siphonariid limpets and the lung-bearing freshwater prosobranchs *Marisa cornuarietis*, *Pomacea paludosa* (Freiburg and Hazlewood, 1977) and *Pomacea lineata* (dos Santos and Mendes, 1981). These amphibious freshwater ampullariids possess both a lung and a gill (Andrews, 1965) and, in common with the intertidal pulmonates, maintain similar levels of oxygen consumption in aerial and aquatic environments (Table 1).

Several species of gill-bearing marine prosobranchs which have a similar distribution on the shore as the siphonariid limpets can also maintain aerobic metabolism at similar levels in air and water (Micallef, 1967; Micallef and Bannister, 1967; Hughes, 1971; McMahon and Russell-Hunter, 1977; Branch and Newell, 1978; Branch, 1979; Houlihan, 1979; Houlihan and Innes, 1982a; Innes, 1982). In contrast to the siphonariids, these prosobranchs are primarily adapted for aquatic respiration and although none possess a specialized lung, gas exchange during aerial exposure may be enhanced in some high shore species by a general increased vascularization of the mantle (Deshpande, 1957; Fretter and Graham, 1962).

The levels of oxygen consumption of some intertidal pulmonate and prosobranch gastropods in air and water are compared in Fig. 3. In accordance with reports by Shumway (1981) and Shumway and Marsden (1982), the respiration rates of gill-bearing pulmonates are very similar to those reported for prosobranchs in both aerial and aquatic environments. It appears that whilst intertidal gill-bearing prosobranchs which lack a specialized lung can maintain gas exchange during aerial exposure at a level similar to that of their pulmonate counterparts (Fig. 3), the converse does not hold true for pulmonates such as *Melampus bidentatus* which do not possess a gill (McMahon and Russell-Hunter, 1975, 1981) (Table 1). In fact *Melampus* possesses many of the physiological characteristics of a fully terrestrial snail but it remains semi-aquatic due to the retention of a veliger larva (McMahon and Russell-Hunter, 1975, 1981).

The difference between aerial and aquatic respiration of *Siphonaria* (Table 1, Fig. 2) can be explained by its high rate of oxygen consumption in air compared to other gastropods (the aquatic rate falls close to that predicted on the basis of other species) (Fig. 3). The elevated aerial rate may be correlated with a larger pulmonary surface area for gas exchange in

Siphonaria. The high aerobic metabolism of *Siphonaria* (Fig. 3) may reflect a high rate of energy turnover compared to other gastropods (Branch and Newell, 1978; Branch, 1979; Newell and Branch, 1980).

The present study complements a previous investigation by Wells and Wong (1978) who concluded that the blood of *Siphonaria zelandica* was better adapted for aerial gas exchange. The oxygen binding properties of the haemocyanin of *Siphonaria zelandica* (Wells and Wong, 1978) and *Amphibola crenata* (Wells and Shumway, 1980) are somewhat similar to those reported for the blood of terrestrial pulmonates (Spok *et al.*, 1964; Wells and Weber, 1982), and in addition amphibious marine pulmonates have the safeguard of an operational reverse Bohr effect for periods of immersion and possible hypoxia (Wells and Wong, 1978; Wells and Shumway, 1980).

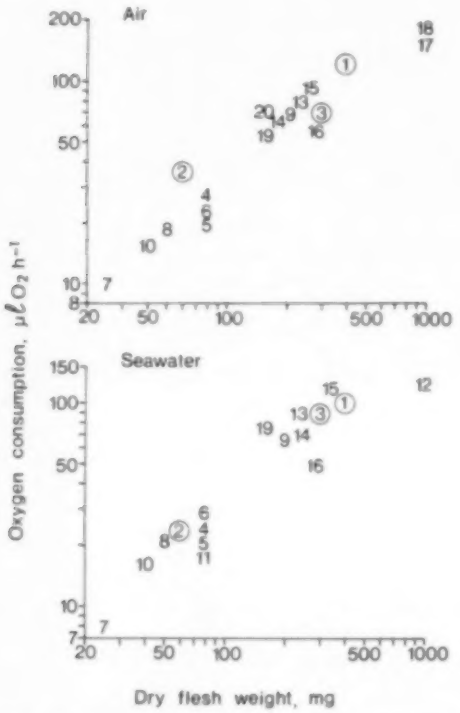

Fig. 3. Rates of oxygen consumption ($\mu l\ O_2\ hr^{-1}$) at 10°C in air and water of standard dry weight (mg) pulmonates (1) *Benhamina obliquata*; (2) *Siphonaria zelandica* (present study); (3) *Amphibola crenata* (Shumway, 1981) compared with a number of prosobranchs; (4) *Littorina littorea*; (5) *Nucella lapillus*; (6) *Gibbula cineraria* (Houlihan *et al.*, 1981; Innes, 1982); (7) *Littorina rudis*; (8) *Littorina obtusata* (Innes, 1982); (9) *Cellana radians*; (10) *Notoacmea pileopsis sturnus* (Innes, in prep); (11) *Thais* (*Nucella*) *lapillus* (Bayne and Scullard, 1978); (12) *Thais lamellosa* (Stickle, 1973); (13) *Patella granatina* (Branch, 1979); (14) *Patella cochlear*; (15) *Patella oculus*; (16) *Patella granularis* (Branch and Newell, 1978); (17) *Patella vulgata*; (18) *Patella aspera* (Davies, 1966b); (19) *Crepidula fornicata* (Newell and Kofoed, 1977); (20) *Littorina irrorata* (Shirley *et al.*, 1978); rates of oxygen consumption of the pulmonates are circled; oxygen consumption of standard weight animals was calculated from the regression analyses and other information provided in the references; conversions from ash free weight and wet weight to dry wegith were made as described by Innes and Houlihan (1981).

The rates of oxygen consumption presented in Fig. 3 were generally made on inactive animals (Houlihan and Innes, 1982a) and differences in the respiratory physiology of gill-bearing pulmonates and prosobranchs may only become apparent when other respiratory indices, e.g. aquatic and aerial aerobic scope for activity (Houlihan and Innes, 1982b), are investigated.

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THE IMPORTANCE OF DISEASE INDUCED CHANGES IN MAMMALIAN BODY TEMPERATURE TO MOSQUITO BLOOD FEEDING

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Abstract—1. Laboratory mice infected with rodent malaria (*Plasmodium berghei* or *P. chabaudi*) or St. Louis encephalitis virus (SLE) were not hyperthermic during the infection period. However, all infected animals displayed pathogen-specific periods of hypothermia.

2. Hamsters infected with *P. berghei* were hyperthermic on day 7 postinfection (PI) but became hypothermic on day 8 PI and remained so until death, approximately 20 days PI.

3. Body temperatures of mice infected with *P. yoelii* were not significantly different from those of uninfected control mice.

4. Mosquitoes (*Aedes aegypti*, *Anopheles stephensi* and *Culex quinquefasciatus*) successfully engorged on restrained, uninfected mice, but were unable to engorge on unrestrained, uninfected mice due to the natural antimosquito behavior of the healthy rodents.

5. Mosquitoes successfully engorged on unrestrained, malaria or SLE infected mice only during certain pathogen-specific periods of infection, but were able to engorge on all restrained, infected mice throughout the infection period regardless of the animal's body temperature.

6. Daily activity patterns of malaria infected mice followed pathogen-specific profiles which closely conformed to the observed mosquito-engorgement profiles.

INTRODUCTION

Hyperthermia is commonly reported in vertebrates infected with arthropod-borne pathogens. Yellow fever virus in African monkeys typically was accompanied by an increase in body temperature of 0.5–3.0°C (Gillett *et al.*, 1950). Even when monkeys suffered inapparent infection, peak viremia often was accompanied by increased body temperature (Gillett and Connor, 1976). Swiss mice infected with Mucambo virus were febrile from day 4 to day 6 postinfection (personal communication, R. E. Shope, Yale Arbovirus Research Unit, New Haven, CT, USA). However, Shope found that feral rodents suffering infections of Guama group virus did not show significant changes in body temperature. Kirn *et al.*, (1967) found body temperature of Sindbis virus infected mice held at room temperature decreased as infection developed. On the other hand, Chernin (1968) reported that malaria-induced lysis of erythrocytes resulted in elevated body temperature in humans.

Several authors have proposed that the warm moist air surrounding vertebrates is an important short-range orientation cue used by blood seeking, hematophagous insects (Wigglesworth and Gillett, 1934; Peterson and Brown, 1951; Gillett, 1971; Davis and Sokolove, 1975). It has also been proposed (Gillett and Connor, 1976; Gillett, 1974) that febrile vertebrates suffering an arbovirus infection become more attractive to host-seeking mosquitoes and that it is thus advantageous for an arbovirus to bring about an increase in host body temperature to "attract" more mosquitoes to these infected hosts.

The purpose of the present study was two-fold: firstly, to determine if rodents infected with *Plasmodium berghei* (Vincke and Lips, 1948), *P. chabaudi* (Landau, 1965), *P. yoelii* (Landau and Killick-Kendrick, 1966), or St. Louis encephalomyelitis virus (SLE) experienced significant changes in body temperature; and secondly, to determine whether any disease-induced changes in body temperature significantly altered mosquito engorgement success on infected animals.

MATERIALS AND METHODS

Body temperature (T_b)

Forty Swiss albino laboratory mice of the same age were separated according to sex and maintained four to a cage. Eight mice were inoculated intraperitoneally with *ca* 10^5–10^6 *Plasmodium berghei* parasites (NK 57 strain) obtained from a donor mouse with a 15% parasitemia. Likewise, eight mice were inoculated with *P. chabaudi* (non-killing strain) and eight with *P. yoelii* (17 X strain). Four mice were given 0.06 ml of St. Louis encephalitis virus (SLE) (virus isolate MSI-7, CDC, Ft. Collins, CO, USA) intranasally. Virus titer was *ca* 7.5 $\log_{10}$ LD_{50}. Finally, 12 mice served as controls and were not treated.

Rectal temperatures of all infected and control mice were monitored with a YSI electronic tele-thermometer (Fisher Scientific Inc.) fitted with a calibrated hypodermic transmitter probe. Temperatures were taken at 0800, 1200, 1600, and 2000 hr daily for the duration of infection (20 days for *P. berghei* and *P. yoelii*, 16 days for *P. chabaudi*, and 8 days for SLE infected mice).

A more sensitive technique was used to measure T_b of *P. berghei* infected hamsters. Six model T mini-mitters (Mini-

Mitter Co., Indianapolis, IN, USA) were coated with Paraffin/Elvax® and surgically implanted into the abdominal cavity of anesthetized hamsters ($N = 6$) of the same age and sex. The temperature-sensitive intermittent oscillation of transmitter telemetry circuits were broadcast as a "click" and received by a walkie-talkie equipped with a beat frequency oscillator. Transmitters were calibrated at a variety of known temperatures in a water bath prior to implantation. A standard curve was made for each transmitter and fitted to a regression line. The T_b of individual animals could then be calculated from the "click rate" of transmitters fit to the individual regression lines. As with mice, T_b of implanted hamsters was taken four times daily. Nine days after implantation, three hamsters were infected intraperitoneally with *ca* 10^5–10^6 *P. berghei* parasites (NK 65 strain). The three remaining hamsters were injected with saline and served as controls. The T_b of all hamsters was monitored as before until infected animals succumbed, *ca* 20 days after infection.

Mosquito blood feeding

Twenty Swiss Albino laboratory mice were infected with *P. berghei* (NK 65 strain) as described above. Once a day, eight mice were placed in individual cages measuring 40 × 22 × 32 cm and exposed for 1 hr to 15, 7–14-day-old, nulliparous *Aedes aegypti* (L.). Within this group of mice, four were free to roam their cage while four were restrained in a flattened, hardware cloth cylinder. Eight uninfected mice were placed in similar cages and exposed to *Ae. aegypti*. Likewise, eight *P. berghei* infected and eight uninfected mice were exposed in overnight tests to *Culex quinquefasciatus* Say (Florida strain) according to the techniques described above for the *Ae. aegypti* experiments. Finally, four *P. berghei* infected and four uninfected mice were restrained and placed overnight into individual cages measuring 40 × 22 × 32 cm containing 15 nulliparous *Anopheles stephensi* Liston.

Twenty laboratory mice were infected with *P. chabaudi* (ANKA Navy strain) and 20 with *P. yoelii* (17 X strain). These mice were exposed to mosquitoes according to the protocol for tests with *Ae. aegypti*, *Cx. quinquefasciatus*, and *An. stephensi* described above for *P. berghei* infected mice.

Eight mice were infected with SLE virus as described above. Once a day infected mice were placed into individual cages measuring 40 × 22 × 32 cm and exposed for 1 hr to 15 nulliparous *Ae. aegypti* (Santo Domingo strain). Four mice were unrestrained and four were restrained. Eight uninfected control mice were similarly exposed to mosquitoes. Mice contracting SLE died 8 days after inoculation. Death due to SLE virus in these animals was confirmed by checking brain smears for virus with an indirect fluorescent antibody technique (Atchson *et al.*, 1966).

All blood feeding experiments were done in a bioroom held at 28°C, 90% RH, and a 16L:8D light regime. At the conclusion of each experiment mosquitoes were aspirated from test cages, counted, and the number of engorged recorded.

Activity of malaria infected mice

An activity cage (Carolina Biological Supply, Burlington, NC, USA) having a wheel fitted with an automatic counter which recorded each half revolution in either direction was used to quantify changes in rodent activity during infection with each malaria species. A 2-month-old, uninfected male mouse weighing *ca* 35 g was placed into the cage 6 days prior to infection. The number of wheel revolutions per 24 hr was recorded daily during this control period. The mouse was then infected as described before with one malaria species and activity was recorded daily during the entire parasitemia. All animals remained in the activity cage for 19 days postinfection. The experiment was replicated five times for each of the three malaria species.

RESULTS

Body temperature (T_b)

Mice infected with *P. berghei* never became febrile. Body temperature remained within the normal range for the first 6 days of infection. After day 6, mice became hypothermic, and remained so until they succumbed to infection *ca* 20 days after inoculation (Fig. 1A). The lowest T_b for infected animals was recorded on days 18–20 postinfection (PI) during which the mean T_b was 29.4 ± 0.8°C. Control mice maintained an average T_b of 36.8 ± 0.3°C for the 20 day test period.

As with mice, hamsters infected with *P. berghei* became hypothermic during later stages of infection. Unlike mice, hamsters were hyperthermic on day 7 of infection when the mean T_b was 39.0 ± 0.6°C as compared with a mean T_b of 37.6 ± 0.3°C for uninfected control hamsters (Fig. 2). Hypothermia in infected hamsters was recorded starting on day 9 PI and persisting until animals died of infection on day 20 PI. Lowest T_b's, with a mean value of 32.2 ± 0.3°C was recorded from day 14 to day 20 PI.

Mice infected with *P. chabaudi* maintained normal T_b for the first 8 days of infection. After day 8, mice

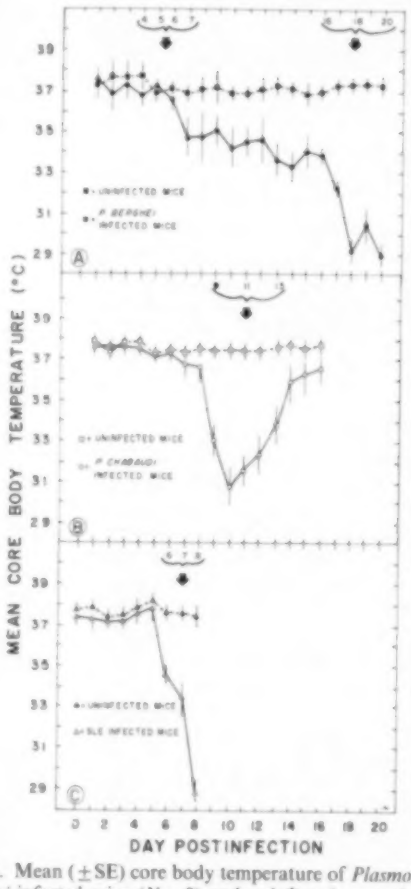

Fig. 1. Mean (±SE) core body temperature of *Plasmodium berghei* infected mice ($N = 8$) and uninfected control mice ($N = 4$) (1A); *P. chabaudi* infected mice ($N = 8$) and control mice ($N = 4$) (1B); and SLE infected mice ($N = 4$) and control mice ($N = 4$) (1C). Arrows indicate days postinfection where mosquitoes successfully engorged on unrestrained, infected mice.

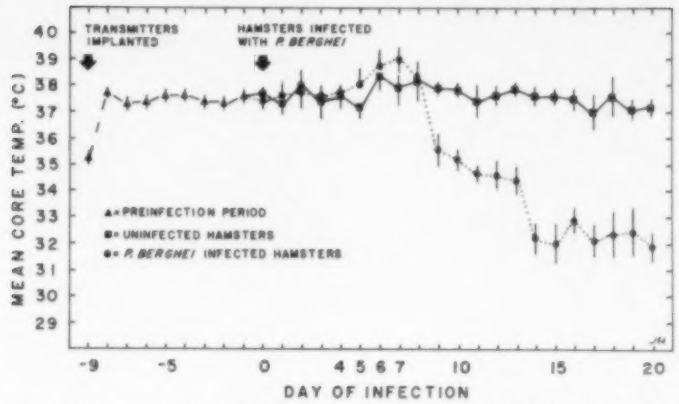

Fig. 2. Mean (±SE) core body temperature of *Plasmodium berghei* infected hamsters ($N = 3$) and uninfected control hamsters ($N = 3$).

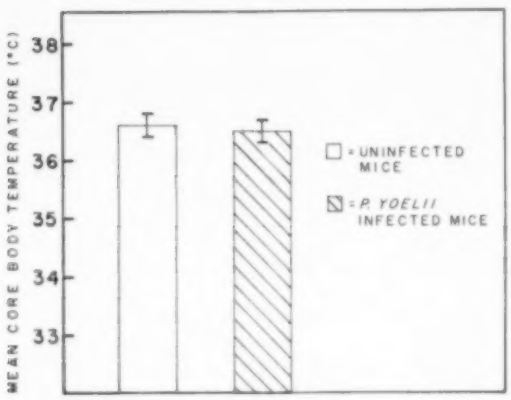

Fig. 3. Mean (±SE) core body temperature of *Plasmodium yoelii* infected mice ($N = 4$) and uninfected control mice ($N = 4$).

became hypothermic. The lowest mean T_b for infected mice was recorded on day 10 PI and was 30.8 ± 2.1°C. After day 10, as mice started to recover from the malaria infection, T_b steadily increased and approached that of control mice on day 16 (Fig. 1B).

The T_b of mice infected with *P. yoelii* did not differ significantly from that of uninfected control mice (Fig. 3).

Mice infected with SLE virus maintained normal T_b until day 6 PI. Beginning on day 6, mice became hypothermic and remained so until death 8 days after virus inoculation (Fig. 1C).

Mosquito blood feeding

Mosquitoes of all three species had great difficulty feeding on unrestrained, uninfected mice and were rarely successful. On the other hand, mosquitoes easily fed on restrained, uninfected mice (Fig. 4).

Mosquito engorgement on unrestrained, infected mice followed pathogen-specific profiles (Table 1).

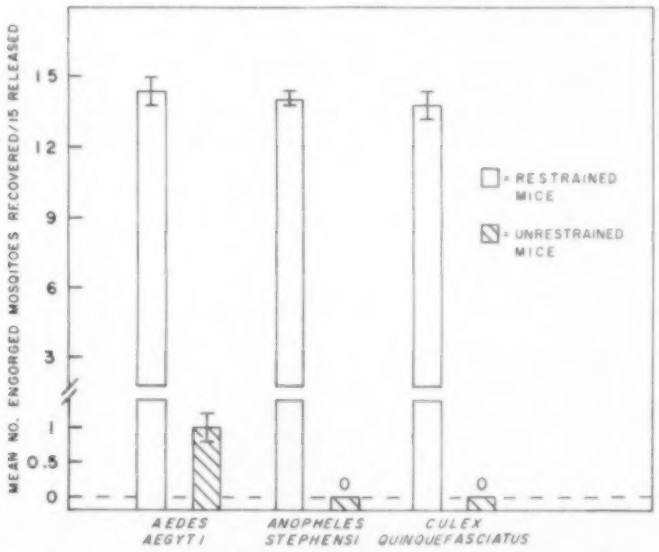

Fig. 4. Mean (±SE) daily engorgement of *Aedes aegypti*, *Anopheles stephensi*, and *Culex quinquefasciatus* on restrained, uninfected mice (open histograms) and unrestrained, uninfected mice (closed histograms). Tests were done for 20 consecutive days (or nights). For each treatment, $N = 4$.

Table 1. Mean daily mosquito blood feeding success (percent of recovered mosquitoes with blood) on unrestrained, infected mice

Pathogen spp.	Mosquito spp.	Percent of mosquitoes with blood — Day postinfection																			
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
P. berghei	*Ae. aegypti*	0	2.0	3.3	10.0	54.7*	72.7*	33.3*	14.7	10.7	0	4.0	1.3	0	4.7	3.3	17.3	30.0*	66.0*	88.0*	86.7*
P. berghei	*C. quinquefasciatus*	0	1.3	6.0	44.0*	54.7*	6.7	4.7	4.7	0	13.3	8.7	6.7	6.7	8.0	0	66.7*	46.7*	73.3*	80.0*	77.3*
P. yoelii	*Ae. aegypti*	1.3	0.6	6.7	6.7	10.7	11.3	38.7*	10.7	5.3	24.7	24.7	14.0	14.0	14.0	6.7	3.3	0	1.3	0	0
P. yoelii	*Cx. quinquefasciatus*	0	0	0	0	0	13.3	26.7*	28.7*	14.7	13.3	17.3	13.3	12.7	5.3	8.7	6.0	6.0	6.0	8.0	6.0
P. chabaudi	*Ae. aegypti*	0	6.0	14.0	1.3	4.7	0	2.0	0	55.3*	60.7*	56.0*	47.3*	33.3*	13.0	4.0	0				
P. chabaudi	*Cx. quinquefasciatus*	6.7	10.0	0	2.0	0	2.7	0	2.0	15.3	53.3*	15.3	5.3	5.3	2.0	2.0	2.0				
SLE	*Ae. aegypti*	10.7	4.7	4.7	0	6.7	66.7*	84.0*	86.7*												

*Period of significant ($P < 0.05$) mosquito engorgement success as estimated by Student's t-test.

With *P. berghei* infected mice, significant numbers of *Ae. aegypti* fed on days 5–7 and 17–20 PI, while *Cx. quinquefasciatus* fed on days 4, 5 and 16–20. With *P. yoelii* infected mice, significant numbers of *Ae. aegypti* fed on day 7 PI while *Cx. quinquefasciatus* fed on days 7 and 8. With *P. chabaudi* infected mice, *Ae. aegypti* successfully fed on days 9–13 while *Cx. quinquefasciatus* fed only on day 10 PI. Finally, *Ae. aegypti* were most successful at feeding on SLE infected mice on days 6–8 PI. With unrestrained infected mice, mosquitoes successfully fed at a variety of T_b values (Fig. 1). The first *P. berghei* feeding peak occurred while the animal's T_b was within the normal range while during the second *P. berghei* feeding peak (days 16–20), the animals were hypothermic. All engorgement on unrestrained, *P. yoelii* infected mice occurred while animals had normal T_b. All engorgement on *P. chabaudi* infected mice occurred while animals were hypothermic (days 9–13 PI). With SLE, significant engorgement also occurred while animals were hypothermic (days 6–8 PI).

When infected animals were restrained and exposed to mosquitoes, engorgement readily occurred throughout the infection period with all pathogens studied (Table 2).

Activity of malaria infected mice

Activity patterns of infected mice were dependent on the malaria species and followed specific profiles. Mean daily activity of mice for the 6-day control period prior to infection with *P. berghei* was 4029 ± 95 cycles per day (Fig. 5A). Activity during days 0–5 PI was variable. A significant ($P < 0.05$) reduction in activity was observed on day 6 and 7 PI followed by a partial restoration of activity on days 8–11 PI. Activity dropped on day 12 and remained low until death occurred on day 20 PI.

Mean daily activity of mice for the 6-day control period prior to infection with *P. chabaudi* was 4441 ± 196 cycles per day (Fig. 5B). Activity patterns of infected animals were variable on days 0–7 PI. Activity dropped significantly ($P < 0.05$) on days 9–17 PI. As mice recovered from the infection, daily activity slowly increased (days 18–19, Fig. 5B).

Mean daily activity of mice during the 6-day control period prior to infection with *P. yoelii* was 4304 ± 786 cycles per day. Activity decreased noticeably on days 5–9 of infection. As mice recovered, mean daily activity increased, reaching normal levels on days 10–19 PI.

DISCUSSION

Elevated body temperature resulting from infection by protozoan or viral agents is common in higher primates and may be evolutionarily adaptive (Kluger, 1978). Little is known about the influence of infectious agents on the body temperature of rodents and small birds. Orsay and Heath (1973) demonstrated that rabbits injected in the pre-optic area with the rodent malaria *P. berghei* suffered hyperthermia. They suggested that malaria parasites produced a pyrogen directly or activated a leukocytic pyrogen upon lysis of infected red blood cells. Little is known about the influence of *P. berghei* on the body temperature of host animals susceptible to infection by

Table 2. Mean daily mosquito blood feeding success (percent of recovered mosquitoes with blood) on restrained, infected mice for the entire infection period

Pathogen	Mean daily percent on mosquitoes with blood during entire infection period (duration of infection)		
	Aedes aegypti	*Culex quinquefasciatus*	*Anopheles stephensi*
P. berghei	98.0 (20 da)	88.7 (20 da)	96.6 (20 da)
P. yoelii	96.7 (20 da)	93.3 (20 da)	91.7 (20 da)
P. chabaudi	96.7 (16 da)	91.3 (16 da)	93.7 (16 da)
SLE	93.3 (8 da)	—	—

this parasite. More is known about influence of body temperature on the course of viral infections in laboratory mice. Lwoff (1959) reviewed studies which showed that when animals with viral infection were kept in artificial hyperthermia (i.e. animals placed in elevated ambient temperatures to increase their body temperature), the virus failed to replicate and animals often recovered. Baron and Buckler (1964) showed that passively induced elevated temperature protected mice suffering from encephalomyocarditis virus even when treatment was begun at maximum virus titer. These authors concluded that non-immune, antiviral factors, such as hyperthermia, were more likely to account for recovery from established viral infection than was antibody. Some animals suffering from malaria routinely enter hyperthermia as the result of parasite produced or induced factors. Animals suffering viral infection also enter hyperthermia as a means of fighting disease.

In the present study, hypothermia, rather than hyperthermia, was observed in mice infected with *P. berghei*, *P. chabaudi* or SLE virus. Hamsters infected with *P. berghei* and implanted with a more sensitive temperature recording device were shown to be hyperthermic for just one day of the infection. As with malaria infected mice, hamsters became hypothermic for the last half of the *P. berghei* infection. The T_b of mice infected with *P. yoelii* did not differ significantly from that of control animals. The physiological reasons for the hypothermia observed in these infected animals are not clear. We observed that rodents suffering from severe cases of malaria (i.e. *P. berghei* and *P. chabaudi*) were anemic and displayed lethargic behavior coincident with hypothermia. However, SLE infected mice, while lethargic, were not anemic during the hypothermic period. Many small rodents have been reported to undergo daily torpor (Lynch *et al.*, 1978). Two prime reasons for torpor in small rodents are food deprivation and cold (Hudson, 1973). It is common for small animals deprived of food to become torpid (Morton, 1978; Wolff and Bateman, 1978; Hudson and Scott, 1979). Animals are able to conserve energy by reducing metabolic rate and entering hypothermia. Experimental animals in the present study were not deprived of food, but during certain periods, as a direct result of infection, they were unable to eat, even when food was within easy reach. Infected animals, too sick to forage, may become hypothermic to conserve energy.

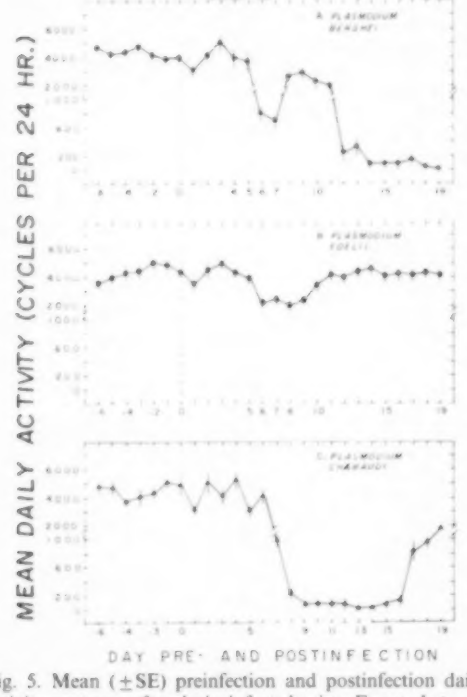

Fig. 5. Mean (±SE) preinfection and postinfection daily activity patterns of malaria infected mice. For each treatment, $N = 5$.

Several authors (Smart and Brown, 1956; Platt *et al.*, 1957; Brouwer, 1960) have suggested that body temperature may be an important stimulus which serves to attract mosquitoes to potential vertebrate hosts. It has also been suggested (Gillett, 1974; Gillett and Connor, 1976; Davis and Sokolove, 1975) that even a small rise in body temperature may be generally attractive or serve to increase body odor and skin moisture which are known to be attractive to host-seeking mosquitoes. In the present study we showed that mosquitoes successfully engorge upon hypothermic animals which, in some cases, displayed body temperature approaching ambient (28°C). *Aedes aegypti*, *Cx. quinquefasciatus* and *An. stephensi* found and fed upon restrained hypothermic mice as quickly as they found and engorged on uninfected, restrained animals with normal T_b values. Mosquito engorgement on unrestrained, infected rodents varied depending on the infective agent and the day post-infection. Elsewhere (Day and Edman, 1983; Day *et al.*, 1983) we have proposed that the time course of rodent susceptibility to mosquito engorgement is pathogen-specific and results from disease induced changes in host anti-mosquito behavior. While it is known that host-seeking mosquitoes are capable of discriminating between different temperature gradients (Davis and Sokolove, 1975; McIver, 1982), it is likely that at best they use host temperature for orientation at distances of less than a meter, or more likely, when they make contact with host animals.

The fact that mosquitoes can quickly locate and feed on hypothermic animals indicates that other short-range cues (perhaps visual and/or olfactory) can be used by host-seeking and blood feeding mosquitoes. It is unlikely that host-seeking mosquitoes differentiate between sick and healthy rodents on the basis of body temperature alone. If there is differential mosquito feeding on these animals in nature, other factors, such as disease induced changes in host defensiveness, are probably responsible.

We report here that mice infected with rodent malaria (*P. berghei* or *P. chabaudi*) or St. Louis encephalitis virus do not suffer hyperthermia, but rather become hypothermic. The body temperature of mice infected with *P. yoelii* does not differ significantly from uninfected control mice. A more sensitive technique for monitoring body temperature in hamsters indicates that animals are slightly hyperthermic on day 7 postinfection but become hypothermic after day 8 and remain so for the duration of infection. Mosquitoes (*Aedes aegypti*, *Anopheles stephensi*, and *Cx. quinquefasciatus*) readily blood feed on animals regardless of their body temperature.

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DAILY ENERGY EXPENDITURE AND WATER FLUX IN BLACK-RUMPED WAXBILLS (*ESTRILDA TROGLODYTES*)

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Abstract—1. Daily energy expenditure ($\dot{H}_{TD}$) and water flux rates were measured in free-flying, aviary-housed *Estrilda troglodytes* using doubly labeled water ($H^3H^{18}O$).

2. Daily energy expenditure of these 6.7 g passerine birds averaged 57.4 ± 15.1 kJ/day (mean ± SD). This is 3.8 times the waxbill's average daytime basal metabolic rate, as determined by respiratory gas exchange with an open-circuit respirometer. We estimate that between 13 and 24% of $\dot{H}_{TD}$ was used to maintain body temperature at night.

3. Daily water efflux averaged 291 ± 36 ml (kg day)$^{-1}$, equivalent to 41% of the total body water pool per day.

4. Daily water influx, 222 ± 58 ml (kg day)$^{-1}$, was 24% lower than efflux, indicating that these small seed-eating birds were unable to meet their water needs without drinking under the conditions of our experiments.

INTRODUCTION

Answers to many fundamental ecological questions regarding birds will require knowledge of water and energy metabolism under field conditions. Unfortunately, because field measurements of metabolism have been difficult to perform, our understanding of avian energy and water metabolism is based largely on two kinds of laboratory measurements: (1) respiratory gas exchange made with small open-circuit respirometers, and (2) food and water consumption made in small cages (for reviews see Calder and King, 1974; Kendeigh *et al.*, 1977). Small chambers or cages severely restrict a bird's behavioral repertoire and data derived from such studies necessarily provide only a hint of what water and energy metabolism might be like under field conditions. We have very little direct information concerning the water metabolism of free-living birds, and what we know of their energy metabolism is derived primarily from time-activity analysis, a technique that is subject to large errors (Weathers and Nagy, 1980). Although water and energy metabolism can be measured directly with doubly labeled water (Lifson and McClintock, 1966), the labeled water technique is sometimes difficult to use in the field because it may be difficult to recapture labeled birds. Large free-flight aviaries would seem to provide a workable alternative to both laboratory and field studies. When appropriately landscaped, aviaries provide semi-natural settings that permit a wider range of behavior than possible in small cages, and the labeled birds can be reliably recaptured. For certain kinds of questions, using doubly labeled water to study the water and energy metabolism of aviary-housed birds may provide a reasonable alternative to field studies. We used this approach to examine water flux and daily energy expenditure ($\dot{H}_{TD}$) in a very small bird—the Black-rumped Waxbill (*Estrilda troglodytes*: family Estrildidae). We were particularly interested in the ratio of $\dot{H}_{TD}$ to basal metabolism, as it is reputedly higher in small birds than in large ones (Walsberg, 1984).

MATERIALS AND METHODS

Animals

The nine birds we used (4 males, 5 females) were obtained from a commercial supplier, but originally had been captured in the wild. They were maintained on a diet of mixed finch seeds, mealworms, and water *ad libitum* for several months before study. We measured their oxygen consumption in the laboratory and their daily energy expenditure in a large outdoor aviary (12 × 6 × 4 m). The aviary, which contained annual grasses about 0.5 m high and several trees, was large enough to permit the birds a wide range of activity and it was thermally heterogeneous enough to provide a range of microclimates. To eliminate complications attributable to variations in the weather, we measured $\dot{H}_{TD}$ of all nine birds simultaneously.

Doubly labeled water

The birds were weighed to the nearest 0.1 g, given an intramuscular injection of 40 μl of water containing ~0.015 mCi ^3H and 95 atom-percent ^{18}O, and held for 1 hr to permit the labeled water to reach equilibrium with body water. We then obtained a 50-μl blood sample from the brachial vein and stored it in a flame-sealed glass microhematocrit tube for later analysis. The birds were then released into the aviary, to which they had been previously habituated. Twenty-four hours later, they were recaptured, reweighed, and a second blood sample obtained. During the measurement period (14–15 May 1981) the birds had access to finch seed, but mealworms and drinking water were not available. Air temperatures and wind speeds at 2 m were recorded hourly at a meteorological station 1 km from the aviary.

The blood samples were micro-distilled (Wood *et al.*, 1975) to obtain pure water that was assayed for tritium activity (Beckman LS 230 liquid scintillation counter, toluene-triton X100-PPO scintillation cocktail) and for oxygen-18 content by cyclotron-generated proton activation of ^{18}O to fluorine-18 with subsequent counting of the gamma-emitting ^{18}F in a

Packard Gamma-Rotomatic counting system (Wood *et al.*, 1975). Isotope measurements were used to calculate body water volumes (^{18}O dilution space), and rates of water influx and CO_2 production using the equations of Lifson and McClintock (1966) as modified by Nagy (1975, 1980).

Basal metabolic rate

The basal metabolic rate of five waxbills was determined during the active phase of their daily cycle by measuring oxygen consumption ($\dot{V}_{O_2}$) with an open-circuit respirometer. For these determinations, the birds were fasted, placed in 4-liter metabolic chambers at 33°C, and given 1 hr to reach thermal equilibrium before $\dot{V}_{O_2}$ was measured. Dry, CO_2-free air passed through Brooks model 5815 mass flow controllers before entering the metabolic chambers. Air flow through the chambers was maintained at 0.25 l min^{-1}. Outlet air was dried, CO_2 removed and the fractional O_2 concentration of inlet and outlet air determined to the nearest 0.005% with an Applied Electrochemistry S-3A O_2 analyzer (N-22M sensor). $\dot{V}_{O_2}$ was calculated from the flow rate (corrected to STPD) and fractional O_2 concentration by equation 2 of Hill (1972).

Accuracy of measurements

Measurements of $\dot{V}_{O_2}$ are within 5% of actual values (Weathers *et al.*, 1980). We did not validate the labeled water technique specifically for use with waxbills, but doubly labeled water measurements of CO_2 production in 11 vertebrates including birds exhibit a mean error of +1.2% (range: −7 to +5%) (Nagy, 1980; Hails, 1979). Validation studies indicate that water flux rates measured with tritium may be expected to be within ±10% of actual flux rates in most situations (Nagy and Costa, 1980).

RESULTS

Total body water volume averaged 70.2 ± 2.5% of body mass (mean ± SD). Rates of CO_2 production and water flux determined by $H^3H^{18}O$ are presented in Table 1. The mean rate of total water efflux (291 ml kg^{-1} day^{-1}) was 31% greater than the rate of water influx (222 ml kg^{-1} day^{-1}). This difference is highly significant statistically ($P < 0.005$, paired two-tailed *t*-test). During the time they were in the aviary, waxbills were provided with finch seeds but not with mealworms or drinking water. Our water flux data reveal that, with two exceptions (Nos 221 and 226), waxbills did not maintain water balance under these conditions. The two birds that did maintain water balance may have obtained additional water by eating insects that were probably present in the aviary, but this is uncertain.

Total $\dot{H}_{TD}$ of the waxbills can be calculated from the CO_2 production data. Doing so, however, requires knowledge of the bird's diet because the caloric equivalent of CO_2 varies with the nature of the substrate being oxidized—ranging from 21.14 kJ/l CO_2 for carbohydrate to 27.25 kJ/l CO_2 for protein and 27.75 kJ/l CO_2 for fat (King and Farner, 1961). Assigning a caloric equivalent to our waxbill's CO_2 production is difficult because we are unsure of their actual diet. During our experiments, the waxbills were provided with a finch-seed mixture containing 78.8% millet, 7.6% rape seed, 6% canary seed, 6% oat groat, 0.8% flax and 0.8% niger. By preferentially selecting certain seeds, the waxbill's dietary intake could have varied from 13.5% protein, 4.5% fat and 71.6% carbohydrate when eating millet, to 22.5% protein, 48.2% fat and 17.3% carbohydrate when eating rape seed (National Research Council, 1971). (The percentages sum to 100% when crude fiber and ash are included.) For a waxbill that was in energy balance, each liter of CO_2 produced would be equivalent to 26.69 kJ of heat if the bird ate rape seed, but only 22.43 kJ if it ate millet (Appendix 1). Because we do not know which seeds our waxbills ate, we assume an average value of 24.56 kJ/l CO_2 for their mixed seed diet.

A further complication arises because in our study most of the waxbills did not maintain body mass, losing an average of 0.68 g during the 24-hr study period. From the water flux data (Table 1), we calculate that 0.46 g of the total mass loss was water. Assuming that the remaining 0.22 g of mass lost was body fat, fat metabolism would account for the production of 0.315 l CO_2 during the 24-hr period (0.22 g fat × 1.433 l CO_2/g fat). This is 13.7% of the total CO_2 production. Assuming that the remaining 86.3% was attributable to the metabolism of seeds, the calculated caloric equivalent for CO_2 produced by our waxbills is 25.0 kJ/l CO_2 (0.137[27.75 kJ/l CO_2] + 0.863[24.56 kJ/l CO_2]). Multiplying this value by the average daily CO_2 production (2.296 l CO_2) gives a mean $\dot{H}_{TD}$ of 57.4 kJ day^{-1}. This is 39% higher than the $\dot{H}_{TD}$ predicted for a 6.7 g bird by Walsberg's (1984) equation, and 3.8 times the measured daytime BMR (Table 2).

DISCUSSION

The Black-rumped Waxbill is one of several closely related seed-eating estrildid finches occurring in Africa. Its range extends in the north from Senegal across sub-Saharan Africa to Ethiopia, thence southward to near the equator. It inhabits open country with thorn scrub, savannas, and scrub and grassy areas near water (Goodwin, 1982). This species has been the

Table 1. Rates of CO_2 production and water flux of Black-rumped Waxbills determined by doubly labeled water

Animal number	Body mass (g)	Water influx (ml · kg^{-1} · day^{-1})	Water efflux (ml · kg^{-1} · day^{-1})	CO_2 production (cm^3 · g^{-1} · hr^{-1})
220	7.1	217	295	19.12
221	6.9	322	322	15.75
222	7.1	195	278	11.32
225	6.4	221	344	18.24
226	7.0	314	314	11.67
227	6.4	152	219	11.15
228	6.7	187	267	8.59
229	6.4	196	298	17.88
230	6.5	198	282	14.79
Mean ± SD	6.7 ± 0.31	222 ± 58	291 ± 36	14.28 ± 3.75

Table 2. Comparison of observed and predicted physiological parameters for Black-rumped Waxbills

Physiological parameter	Predicted*	Observed		
		This study	Lasiewski *et al.* (1964)	Cade *et al.* (1965)
Basal metabolism (kJ/day)	17.4†	15.2	10.9	11.4
Evaporative water loss (ml/kg · day)$^{-1}$)	196 ‡	—	120	—
Ad lib. water consumption (ml/kg · day)$^{-1}$)	—	—	—	412
Water efflux (ml/kg · day)$^{-1}$)	566 §	291	—	—
Daily energy expenditure (kJ/day)	41.2‖	57.4	—	—
Thermal conductance (J/g · hr · °C)$^{-1}$)	7.1¶	—	7.9	7.1
Lower critical temperature (°C)	28.0**	—	28.0	38.0

*For a 6.7 g passerine bird.
†Calculated from the relation kJ/day = 4.56 $g^{0.704}$ for α measurements after Aschoff and Pohl (1970).
‡Calculated from the relation g H_2O/day = 0.432 $g^{0.585}$ from Crawford and Lasiewski (1968).
§Calculated from the relation ml/day = 1.22 $g^{0.590}$ from Degen *et al.* (1982).
‖Calculated from the relation kJ/day = 13.05 $g^{0.605}$ from Walsberg (1984).
¶Calculated from the relation J/(g · hr · °C)$^{-1}$ = 17.2 $g^{-0.463}$ for α measurements after Aschoff (1981).
**Calculated from the relation $T_{lc} = T_b - 9.46\ g^{0.167}$ from Weathers and van Riper (1982).

subject of two prior laboratory studies concerning water and energy metabolism (Lasiewski *et al.*, 1964; Cade *et al.*, 1965). Although these studies used different methods, and obtained slightly different results, they revealed Black-rumped Waxbills to be relatively cold-intolerant and to require water for drinking. Values for physiological parameters from these two studies, together with rates predicted by various allometric equations, provide a convenient basis for analyzing data from the present study (Table 2).

Energy expenditure

Lasiewski *et al.* (1964) and Cade *et al.* (1965) obtained similar values for daytime basal metabolic rate (BMR_a) of resting waxbills. Their values (Table 2) are lower than ours and average only 64% of the rate predicted by the appropriate equation of Aschoff and Pohl (1970). Our BMR value, although higher, is still only 87% of that predicted. A lower than expected BMR could have adaptative value for a very small passerine that, unlike hummingbirds, does not meet the challenges of food shortage by becoming torpid. This parsimonious interpretation is difficult to test empirically, however, and other factors could be involved. For example, small terrestrial endotherms are potentially limited not only by relatively high rates of energy expenditure but also by relatively high rates of water loss. Because a lower than expected BMR also conserves water—through a decrease in ventilation—the ultimate factor favoring the evolution of a low BMR might be the need for water conservation. Given the Black-rumped Waxbill's occurrence in semi-arid thorn scrub, such a scenario is plausible.

Walsberg (1984) derived the following allometric relation for $\dot{H}_{TD}$ of free-living birds: kJ/day = 13.05 $g^{0.605}$ where g is the bird's body mass in grams. This equation predicts that a 6.7 g bird would have a $\dot{H}_{TD}$ of 41.2 kJ/day: a value 28% lower than that which we measured for waxbills in the aviary (Table 2). Thus, although the Black-rumped Waxbill has a low BMR, its $\dot{H}_{TD}$ is relatively high. Walsberg (1980, 1984) noted that because the exponent for $\dot{H}_{TD}$ (0.605) is lower than that for basal metabolism (~0.72), $\dot{H}_{TD}$ is a larger multiple of BMR in small birds than in large ones. For example, the $\dot{H}_{TD}$ predicted for a 10 g passerine bird by Walsberg's equation is 3.1 times BMR_a predicted by Aschoff and Pohl's equation (1970), whereas the predicted $\dot{H}_{TD}$ of a 1000 g passerine is only 1.8 times BMR_a. Small birds are generally more active than large ones and Walsberg (1980) suggested that this accounts for their relatively high $\dot{H}_{TD}$. We have not observed Black-rumped Waxbills in the wild, but in captivity they are very excitable, often flitting about in the vegetation and darting to cover at the slightest alarm. They are obviously more active than larger birds and this undoubtedly partly accounts for their relatively high $\dot{H}_{TD}$. But other factors may also be involved. Of these, one of the most important is probably the relatively high thermoregulatory costs of small birds. Small birds have both higher thermal conductances and higher lower critical temperatures (T_{lc}) than large birds. Thus they incur thermoregulatory costs under environmental conditions that are thermally neutral for large birds. This is illustrated in Fig. 1, which shows the predicted metabolism of a 10 g and a 1000 g passerine bird (curves A and B, respectively) in relation to ambient temperature (T_a). The small bird's metabolism increases when T_a falls below 27°C, whereas the large bird does not begin to experience increased thermoregulatory costs until T_a falls below 11°C. At 0°C the 10 g bird's metabolism is 2.9 times basal, whereas the 1000 g bird's metabolism is only 1.3 times basal. Obviously, even at a fairly moderate T_a a small bird will have a higher $\dot{H}_{TD}$, relative to BMR, than a large bird due to its higher thermoregulatory cost.

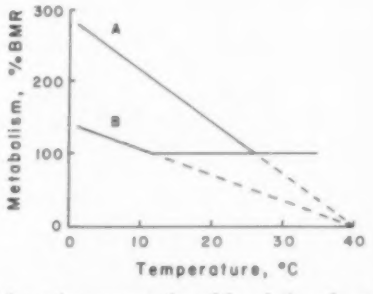

Fig. 1. Schematic representation of the relation of metabolism to temperature in a 10 g bird (A) and a 1000 g bird (B). See text for explanation.

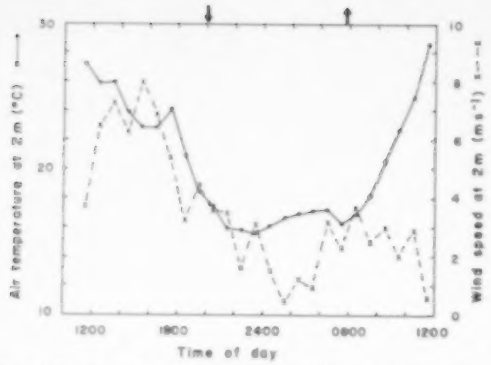

Fig. 2. Relation of air temperature (solid line) and wind speed (dashed line) to time of day during the 24-hr measurement period (14–15 May 1981). Data are from UC Davis meteorological station located 1 km from the aviary. Arrows indicate times of sunset and sunrise.

Determining what proportion of a free-living bird's $\dot{H}_{TD}$ is due to increased thermoregulatory costs is difficult. It requires knowledge of the bird's effective thermal environment and its overall thermal conductance (see Bakken, 1976, 1980; Robinson *et al.*, 1976). In nature the effective thermal environment is determined not just by air temperature but by solar and thermal radiation, wind, humidity, and animal properties such as color, size and shape. We lack the data necessary to calculate our waxbills' true thermostatic requirement, but data for air temperature and wind speed recorded during the 24-hr measurement period (Fig. 2) suggest that thermoregulatory costs comprised a significant portion of their $\dot{H}_{TD}$.

During the 11-hr portion of the 24-hr day when solar radiation did not contribute to the bird's energy budget (19.30 to 06.30 hr in Fig. 2), air temperature averaged 15.8°C and wind speed averaged 2.5 m sec^{-1} (range: 0.4–4.3 m sec^{-1}). The average T_a is 8.8°C below the nighttime T_{lc} predicted for a 6.7 g passerine (Weathers and van Riper, 1982). Both low T_a and wind would have increased the waxbill's rate of heat loss and hence its $\dot{H}_{TD}$. How wind and temperature interact to affect a bird's thermal conductance is poorly known, but based on laboratory data for the American Goldfinch (*Carduelis tristis*; Buttemer, 1981) it seems likely that at 16°C a wind speed of 2.5 m sec^{-1} might have increased the 6.7 g waxbill's thermal conductance by 50%. If so, the waxbill's metabolic rate during the 11-hr "night" would have been 19.0 kJ. (This value is obtained by increasing the bird's predicted thermal conductance (Table 2) by 50% and multiplying it by the difference between mean nighttime T_a (15.8°C) and the bird's assumed body temperature (40°C).) Of the total nighttime energy expenditure, BMR would account for 5.2 kJ. The difference between calculated nighttime metabolism and BMR (19.0–5.2 kJ) gives the thermoregulatory energy requirement: 13.8 kJ. Thus, nighttime thermoregulatory costs alone could account for 24% of the total daily energy expenditure. This approximation may overestimate the true nighttime thermoregulatory cost because it assumes that the birds were fully exposed to the wind. If the waxbills found partial shelter from the wind by roosting on the leeward side of tree trunks, their heat loss would have been less. In the complete absence of wind (an unrealistic assumption), their nighttime thermoregulatory costs would still have been 7.5 kJ: 13% of $\dot{H}_{TD}$. Thus, even under the moderate thermal conditions that prevailed during our study, thermoregulatory costs can account for between 13 and 24% of the waxbill's $\dot{H}_{TD}$. If our measured $\dot{H}_{TD}$ is reduced by 13–24%, the $\dot{H}_{TD}/BMR_a$ ratio would be 3.3 to 2.9. Although these values are still higher than the 1.8 expected for a 1000 g passerine, they indicate that thermoregulatory costs can account for roughly half of the waxbill's relatively higher $\dot{H}_{TD}$.

Water flux

Water flux has been measured in only three species of free-ranging birds: the Song Sparrow (*Melospiza melodia*; Stephenson, 1974), the Phainopepla (*Phainopepla nitens*; Weathers and Nagy, 1980), and the Chukar Partridge (*Alectoris chuckar*; Alkon *et al.*, 1982). Water flux rate is sensitive to body size, short-term variations in diet, meteorological conditions, and available drinking water (see Alkon *et al.*, 1982). Because these factors varied among the three field studies, comparing their measured water flux rates is difficult.

The measured water efflux of waxbills, equivalent to 41% of the body water pool per day, averaged 2.4 times the rate of evaporative water loss found for resting waxbills by Lasiewski *et al.* (1964) (Table 2). Several factors suggest that the water flux rates that we measured represent minimal values for this species under these conditions. First, as noted above, our waxbills were not provided with drinking water during the 24-hr measurement period and, with two exceptions, they were not able to maintain water balance. Presumably they responded to water lack by curtailing water loss. Second, our measured water efflux rate was 29% lower than the rate of *ad libitum* water consumption of caged waxbills measured by Cade *et al.* (1965) (Table 2). Caged birds often waste water if it is provided *ad libitum*, and this may account for the higher consumption rate found by Cade and his coworkers. Third, the least-squares regression lines for water influx and efflux rates in relation to rate of body weight change (Fig. 3) indicate that water balance would be achieved at a water flux rate of 297 ml

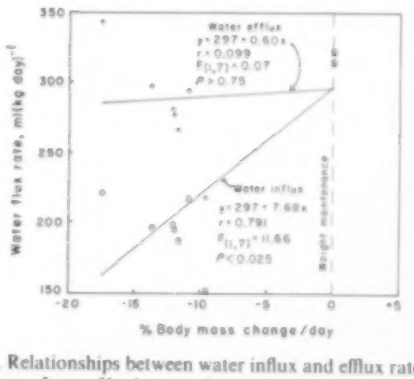

Fig. 3. Relationships between water influx and efflux rates as functions of rate of body mass change. Lines are least-squares regressions.

(kg day)$^{-1}$, nearly the same as the mean water loss rate of 291 ml (kg day)$^{-1}$ (Table 1).

We can estimate the rate of metabolic and dietary water influx these birds would have had if they had eaten just enough seeds to achieve energy balance. The yield of metabolic (oxidative) water for a seed diet is about 0.66 ml H_2O/liter CO_2 produced (Nagy, 1983), so metabolic water production of our waxbills would be about 226 ml (kg day)$^{-1}$. The amount of food needed to provide the energy these birds metabolized (assuming seeds contain 17 kJ/g dry matter and 80% of this energy is available for metabolism) would be 630 g dry matter (kg day)$^{-1}$. If this seed contained 10% water, preformed dietary water input would be 70 ml (kg day)$^{-1}$. Total water input, with no drinking water or insect food, would be 226 + 70 = 296 ml (kg day)$^{-1}$ which is about the same as the mean total water loss in our birds (Table 1). This suggests that our birds could have achieved water balance had they eaten sufficient air-dried seeds to maintain energy balance. We do not know why their feeding rates were lower than this during our measurements, but it might have been due to the abrupt withdrawal of drinking water and mealworms.

The influence of body size on water flux rates can be accounted for by expressing flux on the basis of $kg^{-0.6}$ (Degen *et al.*, 1982). Calculated in this way, the water flux of our waxbills averaged 39.3 ml $kg^{-0.6}$ day^{-1}: a value that falls within the range of water flux rates calculated for five xeric species by Degen *et al.* (1982). Relatively low water flux rates in Black-rumped Waxbills probably reflect the extension of their range into the semi-arid thorn scrub of Africa.

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APPENDIX 1

The caloric equivalent of CO_2 produced from the metabolism of millet is calculated as follows:

(1) Values from the NRC tables for the composition of dry millet seed include crude fiber and ash, and must be converted to a digestible matter basis.

(2) Using data derived from King and Farner (1961) for the caloric equivalent of CO_2, together with the converted values for the composition of millet, 1 gram of dry millet seed yields:

	g × l CO_2/g	= l CO_2	× kJ/l CO_2	= kJ
Protein	0.151	0.653	0.098	27.25	2.67
Fat	0.050	1.433	0.072	27.75	2.00
Carbohydrate	0.799	0.832	0.665	21.14	14.06
			0.835		18.73

Thus, the caloric equivalent for CO_2 production from millet seed is 18.73 kJ/0.835 l CO_2 = 22.43 kJ/l CO_2. The value for rape seed is calculated in the same manner.

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COMPARATIVE STUDIES ON THE ROLE OF CALCIUM IN TRIGGERING SUBCELLULAR DAMAGE IN CARDIAC MUSCLE

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Abstract—1. Isolated cardiac muscle strips from amphibians and mammals, together with isolated frog hearts, have been used as model systems for studying the action of elevated $[Ca^{2+}]_i$ in promoting severe damage.

2. A23187 and caffeine are believed to cause a rise in $[Ca^{2+}]_i$.

3. Elevated $[Ca^{2+}]_i$ causes characteristic damage which has been categorized and includes hypercontraction, Z-line damage and myofilament dissolution. The damage closely resembles that described in the isolated mammalian heart and in skeletal muscle preparations when $[Ca^{2+}]_i$ is raised dramatically.

4. Damage can therefore be triggered by releasing Ca^{2+} from intracellular sites, as distinct from increasing Ca^{2+} entry (as in the Ca^{2+}-paradox).

5. DNP and ruthenium red also cause identical damage and the results suggest that whilst the fall in pH_i associated with ischaemia is probably the consequence of $Ca^{2+}/2H^+$ exchange at the mitochondria, coupled with ATP hydrolysis, lowered pH_i by mitochondrial action is probably not the only cause of myofilament dissolution.

6. Damage is not prevented by pretreatment with leupeptin, an inhibitor of Ca^{2+}-activated neutral proteases, and it is concluded that the latter are probably not implicated in *rapid* and dramatic damage.

7. The possible involvement of lysosomal enzymes in damage triggered by high $[Ca^{2+}]_i$ is discussed.

INTRODUCTION

A high intracellular calcium concentration ($[Ca^{2+}]_i$) may be implicated in myocardial cell death (Katz and Reuter, 1979). Transient episodes of ischaemia are known to cause an acute, marked increase in $[Ca^{2+}]_i$ in cardiac muscle cells (Shen and Jennings, 1972) and Ca^{2+}-channel blockers have been used to preserve the myocardium during ischaemic cardiac arrest (Katz and Reuter, 1979). Results obtained from studies on the "calcium paradox" in cardiac muscle suggest that Ca^{2+}-free perfusion causes sarcolemma damage and that re-introduction of Ca^{2+} allows the massive influx of this ion which, in turn, triggers cellular necrosis (Zimmerman *et al.*, 1967; Crevey *et al.*, 1978; Ashraf, 1979).

We have shown previously that hypercontraction, Z-line sliding and myofilament dissolution can be produced in mammalian and amphibian isolated skeletal muscle preparations by releasing Ca^{2+} from the S.R. by the action of the divalent cation ionophore A23187 (Statham *et al.*, 1976; Publicover *et al.*, 1978) or caffeine (Duncan and Smith, 1978, 1980), from the mitochondria (Duncan *et al.*, 1980a) or by accelerating Ca^{2+}-influx (Publicover *et al.*, 1979). The first objective of the present study was to use amphibian and mammalian cardiac muscle strips (as well as isolated amphibian hearts) as model systems to explore the importance of intracellular Ca^{2+} in initiating myofilament breakdown in heart muscle. To this end, $[Ca^{2+}]_i$ was raised by experimental means that do not damage the sarcolemma and in which the influx of external Ca^{2+} is not involved. The value of developing model systems for the study of cardiac damage has been emphasized previously (McGee *et al.*, 1978). We report here that raised $[Ca^{2+}]_i$ can trigger extensive subcellular damage in cardiac cells.

The Ca^{2+}-paradox is reported not to occur during anoxic perfusion in the absence of glucose, unless the hearts were reoxygenated and it is noteworthy that KCN completely inhibited this oxygen-induced Ca^{2+} paradox. It was concluded that the presence of either ATP or electron transport is necessary for this type of Ca^{2+}-induced damage to occur (both myofilament degradation and the release of creatine kinase); the Ca^{2+} paradox apparently fails to appear in the absence of O_2 but occurs as soon as the electron transport system is reactivated (Ruigrok *et al.*, 1978). Cardiac mitochondria take up Ca^{2+} avidly, releasing protons in exchange, and one possible explanation of these results might be that a marked rise in $[Ca^{2+}]_i$ in cardiac cells is not directly deleterious, but rather that rapid Ca^{2+}-uptake produces localized falls in pH_i which, in turn, promote myofilament dissolution, perhaps via lysosome labilization, since lysosomal breakdown is favoured under acidic conditions (Weissmann, 1965). The second objective of this study has, therefore, been to study the effect of raising $[Ca^{2+}]_i$ when mitochondrial uptake of Ca^{2+} is pre-

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vented and we show that severe damage results even under these conditions.

Finally, we have investigated the effects of an inhibitor of certain Ca^{2+}-activated proteases. A preliminary account of some of these findings has been given previously (Rudge and Duncan, 1980).

MATERIALS AND METHODS

The animals used were mice (Bal/b strain) of 12–20 g and frogs (*Rana temporaria*). Mice were killed by cervical dislocation and the heart was rapidly excised and washed in Krebs–Henseleit solution. Mouse cardiac muscle preparations were prepared by separating the auricle and ventricle and then slicing them into small strips approximately 1 mm in diameter in oxygenated (95% O_2:5% CO_2) Krebs–Henseleit solution at 22°C. Exposure to test agents was carried out in 8 ml of solution at 37°C with continuous oxygenation. Frogs were double pithed and cardiac muscle strips were similarly prepared in Ringer solution without oxygenation and were exposed to test agents in Ringer solution at 22°C. The strips were electrically stimulated where appropriate via external Ag/AgCl electrodes. Isolated frog hearts were prepared for perfusion and recording by conventional techniques. The perfusion saline contained (mM): NaCl 110, KCl 1.88, $CaCl_2$ 1.8, $NaHCO_3$ 1.43, Na_2HPO_4 0.07, glucose 5.6, buffered to pH 7.4 using NaH_2PO_4.

The stock solution of A23187 was dissolved in ethanol; the final concentrations normally used for experiments were A23187 2.5 $\mu g\ ml^{-1}$, ethanol 2.5 $\mu l\ ml^{-1}$. Control solutions contained the same concentration of ethanol.

Muscles were prepared for examination under the electron microscope by replacing the saline with fixative (Karnovsky, 1965) at 21°C for 30 min. Fixation was completed by transfer to a fresh change of the same solution at 21°C for 2½ hr. Specimens were washed in 0.1 M sodium cacodylate with 2.5 mM $CaCl_2$, pH 7.2, for 30 min (two changes). The tissue was postfixed in OsO_4 for 2 hr at room temperature and then washed in cacodylate buffer (two changes), dehydrated through the graded alcohol series and embedded in resin (Spurr, 1969). Sections were cut at 60–90 nm, stained with uranyl acetate and lead citrate (Reynolds, 1963) and examined on an AEI Corinth 500 electron microscope.

Quantitative assessments of the areas of damaged muscle, including mitochondria, were made from electron micrographs as follows. For each treatment, four blocks from each of three repeat experiments were sectioned at three random sites within the block, thus giving a set of 36 grids for each treatment. Randomly selected areas of the electron micrographs were photographed at 2.0 K and printed at 6.8 K. Detailed planimetric measurements of the prints were made on an Apple II microcomputer fitted with a graphics tablet and pen. The computer was programmed for stereometric measurement and calibrated to calculate any area delineated by the pen. The total area occupied by a particular category of damage was expressed as a percentage of the total sectional area examined. Data for control tissue was taken from a set of electron micrographs of sectional areas chosen randomly from all the control tissues from the whole range of experiments that were analysed, so giving one set of control data to which all treatment data were compared.

All reagents were AnalaR grade where these were available. Other reagents were obtained as follows: DNP, ruthenium red, caffeine from Sigma Chemical Co., St Louis; A23187 from Boehringer Corporation Ltd, Lewes, East Sussex; leupeptin from the Peptide Institute Incorporation, Osaka, Japan.

RESULTS

Exposure to normal saline of either frog or mouse control cardiac muscle strips for periods up to 40 min under the conditions described above did not cause subcellular damage and the myofilament apparatus showed no signs of ultrastructural dissolution (Figs 1 and 2).

Patterns of damage

As will be shown, a variety of treatments cause similar patterns of cellular damage in both the amphibian and mammalian preparations. The different types of subcellular degradation can be summarized as follows:

(1) Hypercontracted fibres (Figs 4, 8 and 11).

(2) Extensive blurring of Z-lines (Figs 4, 7 (inset), 9, 10 and 12).

(3) Z-line sliding. This form of damage is common in skeletal muscle but was found only rarely in cardiac muscle in this study. Clear examples were found in mouse ventricle following exposure to DNP.

(4) Disintegration of parts of the Z-line (Figs 4 and 5) showing that damage of this part of the myofilament apparatus also occurred.

(5) Detachment of the myofilaments from the Z-line (Figs 4, 6, 7, 8 and 11).

(6) Extensive myofilament dissolution (Figs 4, 6, 7 and 8) with the myosin filaments being particularly affected (Figs 7, 9 and 12).

(7) Areas in which the myofilament apparatus was completely degraded (Fig. 6).

(8) Marked swelling of the mitochondria with changes in energetic configuration (Figs 7 and 10). Mitochondrial septation also occurred and signs of this can be seen in Figs 5 and 10, but this phenomenon has been reported more fully and discussed elsewhere (Duncan *et al.*, 1980).

These different types of damage agree closely with those described in skeletal muscle following exposure to A23187 or caffeine (Statham *et al.*, 1976; Publicover *et al.*, 1978, 1979; Duncan *et al.*, 1979; Duncan and

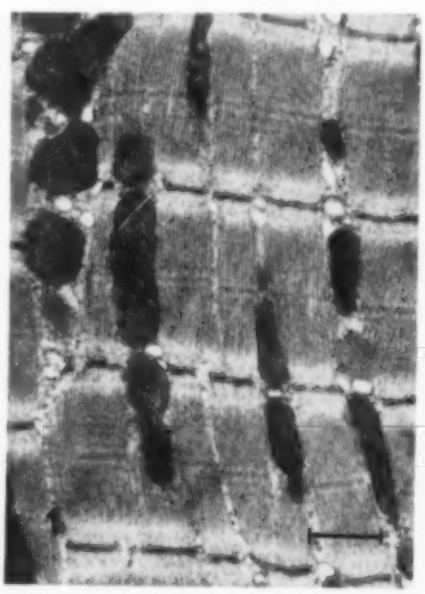

Fig. 1. Mouse, control, 45 min in Krebs solution, pH 7.4, bar = 1 μm.

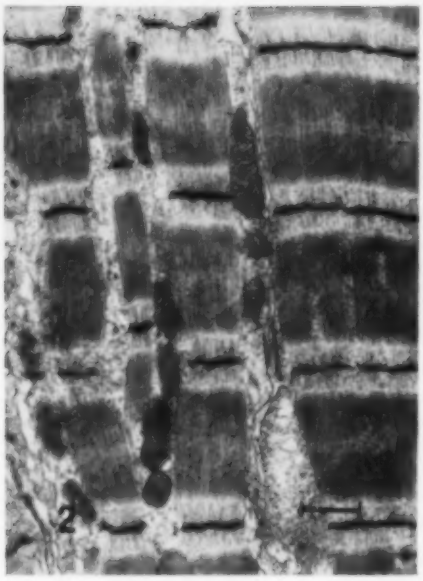

Fig. 2. Frog, control, 40 min in Ringer solution, pH 7.4, bar = 1 μm.

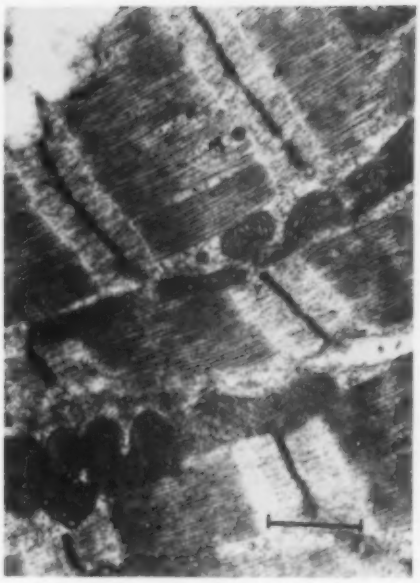

Fig. 3. Frog, A23187 (6.25 μg ml^{-1}) for 45 min, bar = 1 μm.

Fig. 4. Frog, A23187 (2.5 μg ml^{-1}) for 20 min, $[K^+]_o$ being raised to 20 mM for the last 5 min. Arrows show increasing damage to Z-line. Bar = 1 μm.

Fig. 5. Mouse, A23187 (2.5 μg ml^{-1}) for 25 min, for the last 10 min $[K^+]_o$ being raised to 20 mM. Note dividing mitochondrion (arrow). Bar = 2 μm.

Figs 1–12. Electron micrographs of mouse and frog ventricle preparations. Except where stated, preparations are isolated strips; mammalian preparations at 37°C, amphibian at 22°C.

Smith, 1978, 1980; Duncan *et al.*, 1980a) and also correspond with the electron micrographs of cardiac muscle shown in the Ca^{2+}-paradox or the oxygen paradox (Zimmerman *et al.*, 1967; Holland and Olson, 1975; Ashraf, 1979; Singal *et al.*, 1979).

Effect of caffeine on heart strips and on the isolated amphibian heart

Frog and mouse cardiac muscle strips were exposed to 5 or 10 mM caffeine for 20 or 40 min and typical damage was found in both species under all conditions.

Figure 6 illustrates the severe damage found, including damage to myosin filaments, disintegration of the Z-line and an area of complete dissolution. Again, undamaged areas were found. Isolated frog hearts were perfused with 3, 5 or 10 mM caffeine and a marked increase in amplitude was found within 2 min which, at 3 mM, persisted for at least 30 min, although a gradual reduction in rate was detected after 10 min. Hearts fixed while beating after 20 min perfusion of 3 or 10 mM caffeine showed patterns of severe damage identical with those found with heart strips.

Effect of A23187

Unlike skeletal muscle, preparations of amphibian or mammalian cardiac muscle strips were usually unaffected by exposure to the divalent cation ionophore A23187 under comparable conditions (Fig. 3). The following range of treatments was studied: 6.25 and 2.5 $\mu g\ ml^{-1}$ for varying times between 15 and 45 min.

However, extensive ultrastructural damage was found in both amphibian and mammalian cardiac muscle strips if the cells were partially depolarized during exposure to A23187 (2.5 $\mu g\ ml^{-1}$). The preparations were initially exposed to the ionophore for 15 min and extracellular K^+ was then raised to 20 mM for 5–10 min. Similar results were obtained if the strips were made to contract by field stimulation. Under these conditions severe myofilament dissolution (see Figs 4 and 5) was found although undamaged areas of muscle were still found sometimes, even in examples of extreme damage.

A similar pattern of damage was found in the electron micrographs of amphibian and mammalian cardiac muscle strips. Figure 5 shows disrupted mouse tissue with Z-lines largely unaffected but with evidence of myofilament dissolution. One mitochondrion shows a transverse septum. Figure 4 is of frog cardiac muscle and illustrates a spectrum of damage; part of the myofilament apparatus is hypercontracted with Z-line blurring whilst another shows a sequence of damage of the Z-line (from being almost intact to only the remnants being detectable) which is accompanied by the progressive loss of the organisation of the myofilament apparatus.

The damage observed seems to be irreversible; return to normal saline for up to 60 min did not produce any clear signs of recovery.

Effect of mitochondrial uncoupling with DNP

Muscle strips were exposed to 10^{-4}M, 2,4-dinitrophenol (DNP) for 25 min to produce mitochondrial uncoupling, and were then transferred to 10^{-4}M DNP plus A23187 (2.5 $\mu g\ ml^{-1}$) for a further 25 min. Electron micrographs of both amphibian and mammalian muscles showed that the same extensive damage still occurs. Indeed, exposure to DNP alone (10^{-4}M or 10^{-5}M; 10–25 min) itself caused major myofilament dissolution, accompanied by mitochondrial swelling and division. Mouse cardiac muscle is shown in Fig. 7 which shows intense blurring of Z-lines and the dissolution of the myosin filaments. Similar types of damage were found in frog cardiac muscles. Figure 8 shows a range of different types of damage; areas of hypercontraction with heavily blurred Z-lines, separation of the myofilaments from the Z-line, Z-line loss and disintegration of the myofilament apparatus.

Fig. 6. Frog, 5 mM caffeine for 20 min. Bar = 1 μm.

Such findings following DNP treatment when the mitochondria would have been uncoupled suggest that $Ca^{2+}/2H^+$ exchange across the mitochondrial membrane is probably not a necessary prerequisite for the induction of cellular damage.

Ruthenium red

Ruthenium red passes across the sarcolemma of cardiac muscle (Forbes and Sperelakis, 1979) and

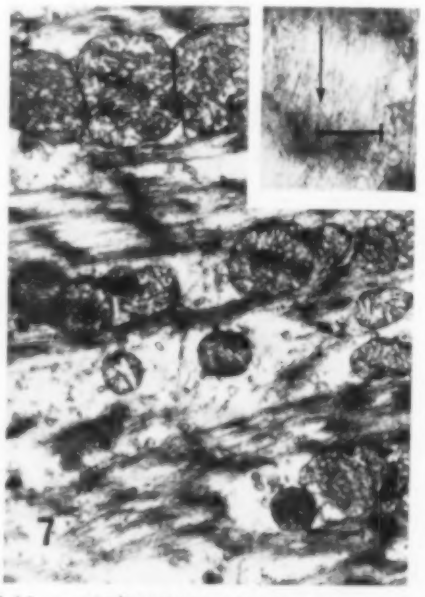

Fig. 7. Mouse, 10^{-4}M DNP for 25 min; bar = 1 μm. Inset shows another preparation where the damage is primarily to the myosin filaments. Arrow shows blurring of the Z-line. Bar = 0.5 μm.

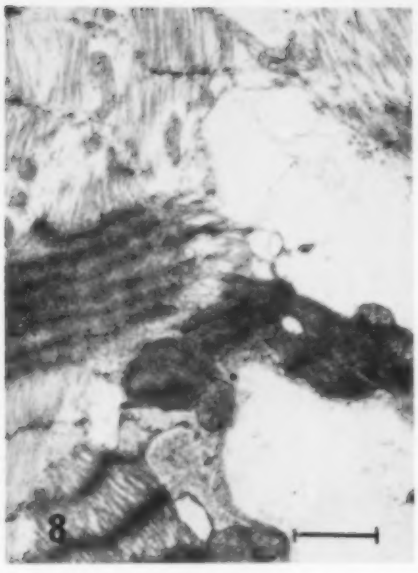

Fig. 8. Frog, 10^{-4}M DNP for 25 min. Bar = 2 μm.

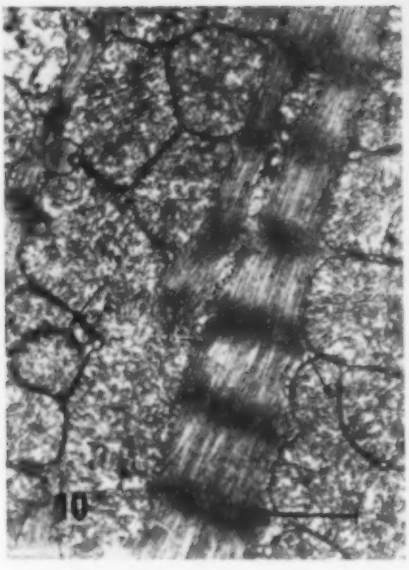

Fig. 10. Mouse, 6 μM ruthenium red for 40 min. Note swollen and dividing mitochondria (arrow). Bar = 1 μm.

specifically inhibits mitochondrial uptake of Ca^{2+}. Exposure of either amphibian or mammalian cardiac muscle strips to 6 μM ruthenium red for 20 or 40 min followed by A23187 (2.5 μg ml^{-1}; 10 min) plus ruthenium red again produced typical subcellular damage; Fig. 9 shows Z-line blurring and the disappearance of myosin filaments in frog muscle. Ruthenium red (4 or 6 μM for 20, 40 or 60 min) alone also caused subcellular damage in amphibian or mammalian preparations (Fig. 10; note swollen mitochondria and transverse septa).

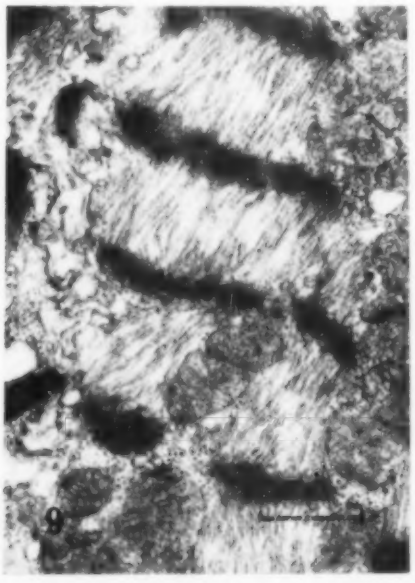

Fig. 9. Frog, 6 μM ruthenium red for 40 min with A23187 (2.5 μg ml^{-1}) added for the last 15 min. Bar = 1 μm.

Quantitative assessment of damage

A summary of the data obtained from the computer graphics analysis of the electron micrographs is shown in categories (a) to (i) which broadly represent increasing severity of damage. Electron micrographs of control experiments showed more than 99% of the sarcomeres in category (a). Categories (j) to (l) illustrate increasing degrees of mitochondrial abnormality; control sections show mitochondria almost exclusively in category (j). Category (m) covers various intra- and intercellular spaces and capillaries.

The four different treatments studied (A23187, ruthenium red, caffeine, DNP) all produced very similar patterns of damage to the myofibrils in both frog and mouse preparations, with the greatest areas being hypercontracted. Damage was more severe in the mouse preparation.

The four treatments also caused extensive changes in mitochondrial ultrastructure from the normal condition (j). Mouse mitochondria were more severely affected, the majority of the organelles showing changes. The ruthenium red treatment (at the concentration and time of exposure chosen) appears to be the most effective in producing mitochondrial swelling.

Mitochondrial swelling, frequently accompanied by the formation of septa and subdivision of the organelles, commonly accompanies myofibrillar damage in skeletal and cardiac muscle, and these phenomena have been discussed previously (Duncan *et al.*, 1980b). Figure 13 illustrates clearly the overall effect of these treatments that result in myofibrillar damage; myofibrillar area decreases whilst mitochondrial area increases because of swelling and division. In control preparations of both mouse and frog tissue, myofibrillar : mitochondrial area was 2 : 1 whilst this ratio changed significantly to nearly 1 : 1 after many treatments ($P < 0.001$ in the majority of experiments; Student's t-test; Fig. 13). Total mitochondrial area

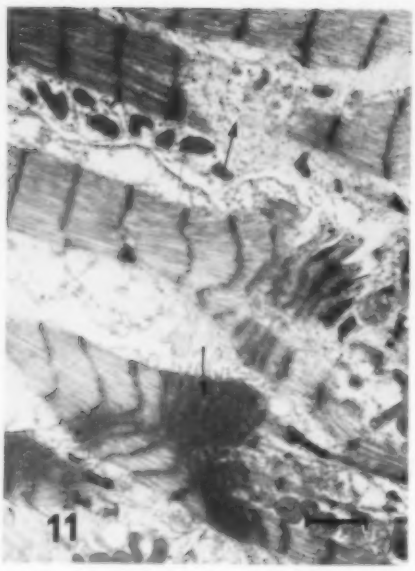

Fig. 11. Frog heart perfused with Ringer solution containing 8×10^{-5}M DNP for 18 min and fixed after perfusion with normal saline for a further 50 min. Arrows show hypercontraction and myofilament loss. Bar = 2 μm.

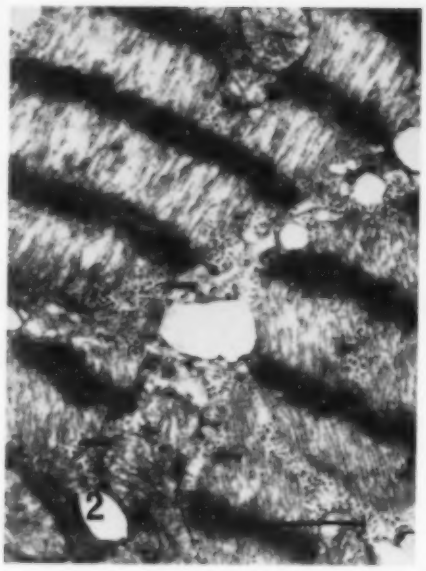

Fig. 12. Frog heart perfused with Ringer solution containing 10^{-5}M DNP and fixed after 120 min. Bar = 1 μm.

exceeded the area of myofibrils in mouse cardiac muscle after exposure to A23187.

Isolated amphibian hearts perfused with DNP or ruthenium red

Perfusion with Ringer containing 10^{-4}M DNP caused contractile activity in isolated frog hearts to stop in 3–5 min, and electron microscopy of the muscle at this stage again revealed typical myofilament degradation. Most preparations did not recover when reperfused with normal saline. Perfusion with Ringer containing 6×10^{-5}M or 8×10^{-5}M DNP also caused the heart to stop beating, but it generally recovered when perfused with normal saline. Again examination of electron micrographs fixed during reperfusion with normal saline for periods up to 160 min revealed extensive myofilament dissolution which appeared to be identical with that found in isolated muscle strips. Figure 11 illustrates hypercontraction and myofilaments separating from the Z-line, and Fig. 12 shows the loss of myosin filaments together with Z-line blurring. Hearts perfused with 10^{-5}M DNP continued beating but, once more, examination showed identical damage in many parts

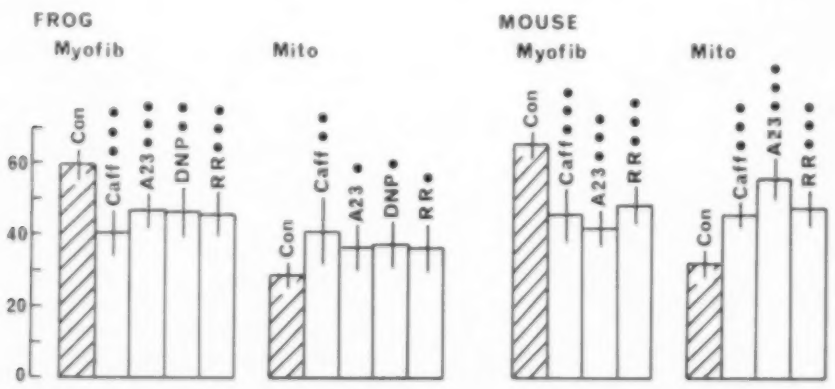

Fig. 13. Effect of various treatments on the relative areas of myofibrils and mitochondria in frog and mouse cardiac muscle strips. Ordinate: percentage of total area occupied, as determined on a graphics computer. Results analysed by Student's *t*-test: significance, one circle $P < 0.05$, two circles $P < 0.01$, three circles $P < 0.001$. Conditions: caffeine 10 mM, 30 min; A23187 2.5 μg ml^{-1}, 15 min. ($[K^+]_o$ = 20 mM); DNP 10^{-4}M, 25 min; ruthenium red 4 μM, 40 min. Note that the effects of the different treatments on the relative areas of the major components are very similar in both species and that damage is always accompanied by a reduction in myofibril area and an increase in mitochondrial area.

Table 1. Percentage areal density of various categories of the tissue components in sections of frog ventricular strips after exposure to various treatments. Component and total areas were measured on a graphics computer

Category	A23187	Ruthenium red	Caffeine	DNP
Myofibrils				
(a) Relaxation of sarcomeres	2.5	5.3	8.2	4.9
(b) Relaxation of sarcomeres with some myofilament loss	6.2	1.4	1.1	4.8
(c) Contraction of sarcomeres	0	0.9	1.5	4.0
(d) Contraction of sarcomeres with some myofilament loss	8.1	0	1.2	1.7
(e) Hypercontraction of sarcomeres	0	5.6	1.2	2.5
(f) Hypercontraction of sarcomeres with some myofilament loss	2.7	0	0.7	6.2
(g) Extensive hypercontraction of sarcomeres	21.4	26.6	16.1	18.2
(h) Clumped contraction bands	5.1	3.9	9.6	4.0
(i) Dissolution/teased out myofilaments	0.9	1.8	0.8	0.3
Mitochondria				
(j) Regular cristae and small matrix spaces	17.3	2.1	17.5	15.9
(k) Regular cristae and slightly enlarged matrix spaces. Mitochondria slightly swollen	19.4	32.7	7.8	15.9
(l) Mitochondria swollen. (Matrix clearing to various extents; fragmentation of cristae may be present.)	0	1.6	15.4	6.0
(m) Spaces	11.2	14.7	13.8	12.4
(n) Nucleus	5.3	3.3	5.0	3.2
N	16	17	19	18

Caffeine 10 mM, 30 min; A23187 2.5 μg ml^{-1}, 15 min ($[K^+]_o$ = 20 mM); DNP 10^{-4} M, 25 min; ruthenium red 4 μM, 40 min.

of the tissue. Although areas of hypercontraction were seen, it was noteworthy that no marked increase in amplitude or frequency of beat was seen in these experiments with DNP.

Hearts perfused with 6 μM ruthenium red for 18 min before fixation also showed the same extensive ultrastructural damage, although in most experiments the heart was still beating strongly at this time. As with DNP, no increase in the amplitude or frequency of beat was detected during the perfusion.

Action of leupeptin

Leupeptin is believed to be able to enter muscle cells and is an inhibitor of Ca^{2+}-activated neutral proteases (Duncan *et al.*, 1979). Its action in possibly protecting against the damage induced by the treatments described above was tested in a series of experiments. Concentrations of leupeptin used were 45 and 90 μM, and frog and mouse cardiac muscle strips were pretreated with the inhibitor for 40 min before exposure to DNP or to A23187 when the muscles were partially depolarized with elevated $[K^+]_o$, as described above. In all experiments, no protection was apparently afforded by pretreatment with leupeptin; extensive myofilament degradation was found and it was noteworthy (see below) that the Z-lines showed the usual damage.

Table 2. Percentage areal density of various categories of the tissue components in sections of mouse ventricular strips after exposure to various treatments. Component and total areas were measured on a graphics computer

Category	A23187	Ruthenium red	Caffeine
Myofibrils			
(a) Relaxation of sarcomeres	0	0.2	0.1
(b) Relaxation of sarcomeres with some myofilament loss	0.1	0	0
(c) Contraction of sarcomeres	2.1	0	1.2
(d) Contraction of sarcomeres with some myofilament loss	0	0	0
(e) Hypercontraction of sarcomeres	0.4	1.4	0.2
(f) Hypercontraction of sarcomeres with some myofilament loss	0	0.6	17.8
(g) Extensive hypercontraction of sarcomeres	13.1	29.0	5.0
(h) Clumped contraction bands	23.6	16.8	17.4
(i) Dissolution/teased out myofilaments	2.0	0.6	4.5
Mitochondria			
(j) Regular cristae and small matrix spaces	5.8	2.3	9.7
(k) Regular cristae and slightly enlarged matrix spaces. Mitochondria slightly swollen	46.6	31.3	35.1
(l) Mitochondria swollen. (Matrix clearing to various extents; fragmentation of cristae may be present.)	3.7	14.3	1.2
(m) Spaces	0.9	3.4	6.3
(n) Nucleus	1.3	0	1.9
N	19	16	20

Caffeine 10 mM, 30 min; A23187 2.5 μg ml^{-1}, 15 min ($[K^+]_o$ = 20 mM); DNP 10^{-4} M, 25 min; ruthenium red 4 μM, 40 min.

DISCUSSION

Ca^{2+} *and subcellular muscle damage*

Amphibian and mammalian cardiac muscle strips appear to be a suitable model system for studying the cellular events associated with myofilament degradation. The patterns of damage seen following the different treatments described are very similar, both qualitatively and quantitatively, and also correspond closely with those described previously in skeletal muscle preparations (Statham *et al.*, 1976; Duncan, 1978; Publicover *et al.*, 1978, 1979; Duncan *et al.*, 1980a) and in isolated mammalian hearts (Holland and Olson, 1975; Ashraf, 1979; Singal *et al.*, 1979). In particular, the different types of damage described above correspond closely with those seen in electron micrographs of isolated rat heart following anoxic perfusion where Z-line blurring and myofilament dissolution can all be seen, and close inspection of the figures presented by Ganote *et al.* (1975) reveals that not only are the mitochondria swelling but that some are developing transverse septa, as we have described previously in isolated cardiac muscle strips (Duncan *et al.*, 1980b) and is shown in Fig. 10. Damage develops very quickly in all these examples.

The studies with skeletal muscle have shown that severe damage follows rapidly when $[Ca^{2+}]_i$ is raised markedly with such treatments as A23187. The present results support the hypothesis that rises in $[Ca^{2+}]_i$ can also trigger cellular damage in cardiac muscle; the similarity of the damage (both Z-line blurring and the myosin filaments being particularly affected, see below), the hypercontracted fibres and the swollen mitochondria (Publicover *et al.*, 1978) all suggest that the concentration of Ca^{2+} in the cytosol has risen markedly.

Caffeine is known to induce contraction in cardiac muscle by the release of Ca^{2+} from the S.R. (Chapman and Miller, 1974) and it promotes the Ca^{2+}-induced release of Ca^{2+} from the S.R. of skeletal muscle, where it triggers typical degradation of the myofilament apparatus in amphibian and mammalian muscle (Duncan and Smith, 1978, 1980). A Ca^{2+}-induced release of Ca^{2+} has now been described in amphibian and mammalian heart muscle (Fabiato and Fabiato, 1978). Caffeine consistently caused an increase in amplitude of beat in isolated frog hearts in the present study and we conclude that the subcellular damage in cardiac muscle strips and in isolated hearts that was found in the present study is also the direct consequence of a marked rise in $[Ca^{2+}]_i$. Ca^{2+} enters across the sarcolemma in the calcium paradox, $[Ca^{2+}]_i$ rises, so initiating damage. The present study shows that a similar pattern of damage can also be triggered by releasing Ca^{2+} from intracellular sites.

In the light of these findings it is, at first sight, surprising that the divalent cation ionophore A23187 alone does not induce severe myocardial damage. It causes dramatic damage in skeletal muscle. It increases the force of contraction, facilitates $^{45}Ca^{2+}$ entry and causes the release of intracellularly stored Ca^{2+} in isolated guinea pig atrium (Holland *et al.*, 1978). However, presumably $[Ca^{2+}]_i$ rises to sufficiently high levels to trigger massive myofilament dissolution in cardiac muscle only when the action of A23187 at the S.R. is augmented by the Ca^{2+}-influx following the increase in P_{Ca} associated with depolarization. We suggest that these differences in the response to A23187 reflect the relative importance of the systems that control $[Ca^{2+}]_i$ and provide the source of Ca^{2+} at excitation; the S.R. is of particular importance in skeletal muscle, whereas extracellular Ca^{2+} is involved in the excitation of cardiac muscle.

DNP and ruthenium red

Both these agents rapidly trigger identical damage in cardiac muscle; both are potentially able to release Ca^{2+} from the mitochondria by uncoupling (DNP) or by inhibiting Ca^{2+}-uptake (ruthenium red). The mitochondria constitute a major intracellular Ca^{2+}-store in cardiac muscle and it is tempting to suggest that both agents act by releasing Ca^{2+} from the mitochondria. However, neither causes an increase in the amplitude or frequency of heart beat and it must be concluded that, if the mitochondrial stores are depleted, $[Ca^{2+}]_i$ is not rising significantly at the myofilament apparatus.

Intracellular pH

There is considerable evidence that changes in pH_i are associated with myocardial damage. Ischaemia causes a fall in pH_i and irreversible cell injury has been reported when the interstitial pH falls below 6.3; pH_i falls as a function of anoxic time, and morphological changes correlate well with falling pH_i (Gebert *et al.*, 1971; Schaper and Knoll, 1979).

Cardiac muscle mitochondria are known to accumulate Ca^{2+} in exchange for protons, the stoichiometry being $Ca^{2+}/2H^+$ (Williams and Fry, 1979). Furthermore, the injection of $CaCl_2$ into snail neurones causes an immediate fall in pH_i, the injected Ca^{2+} being exchanged for H^+ at the mitochondria. If this uptake process is blocked by ruthenium red, the Ca^{2+} is taken up by a second, much slower process which also releases H^+ (Ahmed and Connor, 1980; Meech and Thomas, 1980). It therefore seems probable that the falls in pH_i recorded during ischaemia, in spite of the substantial buffering capacity of cardiac cells (Ellis and Thomas, 1976), are the consequence of mitochondrial Ca^{2+}-uptake as the organelles attempt to correct the massive influx of extracellular Ca^{2+} (Hearse *et al.*, 1978) and of the hydrolysis of ATP.

One possibility is that the fall in pH_i itself is responsible for cardiac damage (Duncan *et al.*, 1980a), perhaps by promoting lysosomal labilization which is favoured by acidic conditions (Weissmann, 1965). In possible support of this view, inhibition of electron transport by KCN has been reported as completely preventing the oxygen-induced Ca^{2+}-paradox (Ruigrok *et al.*, 1978), perhaps suggesting that it is the re-uptake of Ca^{2+} that triggers cellular damage. However, the present experiments show that damage still ensues when the mitochondria are uncoupled with DNP or (more significantly) when Ca^{2+}-uptake is prevented by ruthenium red, suggesting that a fall in pH_i associated with Ca^{2+}/H^+ exchange at the mitochondria is not the only mechanism triggering damage, although pH_i may still fall in the presence of these inhibitors as the consequence of Ca^{2+}/H^+ exchange at other intracellular binding sites and because of ATP hydrolysis, and thereby contribute to impaired myocardial functioning.

Ca^{2+} *and the triggering of myofilament degradation*

High $[Ca^{2+}]_i$ induced by caffeine or A23187, could promote the dissolution of the myofilament apparatus in at least two different and separate ways:

(a) Ca^{2+}-activated proteases from skeletal muscle have been extensively studied; they are free in the cytoplasm, act relatively slowly and are inhibited by leupeptin. Their targets are limited but include the Z-bands and troponins I and T (see summary, Duncan, 1978), although they may have a greater effect on the myofibrils than hitherto supposed (Azanza *et al.*, 1980). A similar protease is present in mammalian cardiac muscle (Toyo-Oka and Masaki, 1979; Dayton and Schollmeyer, 1980). Although these proteases might contribute to Ca^{2+}-triggered damage, especially at the Z-lines, it seems that their action is too slow and their targets are too limited for them to account for the range of damage described in the present study. Furthermore, the present results show that damage is not reduced and that the Z-lines still show blurring and disintegration following exposure to leupeptin, an inhibitor of Ca^{2+}-activated proteases. The lowest concentration of leupeptin and the times of preincubation chosen correspond with those used to reduce or prevent protein degradation in normal and diseased skeletal muscle (Libby and Goldberg, 1978). Leupeptin similarly failed to prevent the damage triggered by A23187 in skeletal muscle (Duncan *et al.*, 1979), and we conclude that the Ca^{2+}-activated proteases do not have a major role in causing the *rapid* damage (including that at the Z-lines) in either cardiac or skeletal muscle.

(b) The evidence suggests, therefore, that there is a second, Ca^{2+}-triggered process that is primarily responsible for the degradation of the myosin filaments. Experimental evidence suggests that lysosomal enzymes may be involved (Kennett and Weglicki, 1978; Decker *et al.*, 1979; Wildenthal and Crie, 1980), and cathepsins B and D are of particular interest since they are probably the only enzymes present in the cardiac muscle cell that can degrade the myofilaments (Bird *et al.*, 1980). Marked rises in $[Ca^{2+}]_i$ might promote lysosomal breakdown by a hitherto unidentified mechanism and any concomitant falls in pH_i would accelerate the action of lysosomal enzymes released into the cytosol. The hypercontracted fibres seen in the present study suggest a high $[Ca^{2+}]_i$ and ATP consumption (which has been recorded in cardiac muscle damage, Boink *et al.*, 1976; Ruigrok *et al.*, 1978; Watts *et al.*, 1980), whilst myofilament dissolution, with the myosin filaments being particularly affected, suggests the action of enzymes such as lysosomal cathepsins.

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CHEMOATTRACTANTS OF THE SHRIMP, *PALAEMONETES PUGIO*: VARIABILITY IN RESPONSIVENESS AND THE STIMULATORY CAPACITY OF MIXTURES CONTAINING AMINO ACIDS, QUATERNARY AMMONIUM COMPOUNDS, PURINES AND OTHER SUBSTANCES

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Abstract—1. In contrast to earlier findings, a crab extract was found to be a more potent attractant of the shrimp, *Palaemonetes pugio*, than a synthetic mixture containing the amino acids and betaine in the extract.
2. This difference was not attributable to changes in responsiveness induced by feeding state or time of year.
3. A synthetic mixture with a stimulatory capacity similar to the crab extract was produced by incorporating the following additional extract constituents: purines and purine nucleotides, homarine, trimethylamine oxide and lactic acid.
4. Adenosine 5′-monophosphate was the most potent single substance in the mixture.
5. A synergistic effect between components was indicated.

INTRODUCTION

In an earlier study of chemoattractants for the marine shrimp, *Palaemonetes pugio*, mixtures of amino acids plus the related substance, betaine, were found to comprise the major attractants present in aqueous extracts prepared from three organisms (Carr, 1978). However, recent work with *P. pugio* revealed that mixtures of amino acids plus betaine did not account for the total activity of the natural extracts. Indeed, the responses to the above mixtures were lower than observed previously. Hence, a study was initiated to account for the differences between the recent findings and the earlier ones. This study focused on two general questions. (1) Do factors such as seasonal change or feeding state cause major changes in the relative responsiveness of shrimp to a natural extract and a synthetic solution? (2) What substances, in addition to the amino acids and betaine, make significant contributions to the chemoattractive capacity of a natural extract?

This report shows that responsiveness of the shrimp is affected by both time of year and feeding state. Further, it is shown that some or all of the following substances make important contributions to the chemoattractive capacity of an aqueous crab extract: amino acids, betaine, purines and purine nucleotides, homarine, trimethylamine oxide and lactic acid.

MATERIALS AND METHODS

The following procedures were adapted from those previously reported (Carr and Gurin, 1975; Carr, 1978) to provide a very rigorous standardization of both the condition of the test animals and the procedures for bioassay.

Maintenance of animals

New lots of *Palaemonetes pugio* were collected at monthly intervals from the estuarine habitat near the Whitney Laboratory. Groups of about 300 non-gravid individuals were added to each of five 51 × 41 × 22 cm polycarbonate aquaria provided with running, full-strength sea water and aeration. Before use in experiments, all new groups of shrimp were maintained in the laboratory for an acclimation period of two weeks with a daily feeding of TetraMin (TetraWerke, Melle, Federal Republic of Germany). Four days before the first bioassays, the following schedule of feeding, cleaning and testing was begun and continued throughout the time the group was utilized:

Day 1. Feed group 5 g TetraMin at *ca* 4–5 p.m. This is enough food for group to feed *ad libitum* until the next day.
Day 2. Clean all uneaten food and debris from aquarium.
Day 3. (= Day one, post-feed) Leave shrimp undisturbed.
Day 4. (= Day two, post-feed) Use shrimp in assays between 10 a.m. and 4 p.m. Feed group 5 g TetraMin after completion of testing.
Days 5, 6 and 7. As in Days 2, 3 and 4 above.

By maintaining five separate tanks of shrimp and staggering the feeding schedules, one or more tanks were ready for testing each day. For the tests of the effect of feeding state on responsiveness, the shrimp were not fed after Day 4 (= Day two, post-feed).

Bioassay procedures

Bioassays were conducted at room temperature (21.9–25.6°C) in transparent plastic boxes (31 × 17 × 4 cm) divided by partitions into six compartments of equal size. Three shrimp were added to 140 ml of sea water in each compartment to provide groups of 18 shrimp per box (= one test group). Each box was housed inside a wooden frame covered with a sheet of grey-tinted plexiglass. Holes in the sheet received a No. 00 rubber stopper bored to accomodate the delivery tube (2.4 mm ID Tygon) that passes through the stopper and on into the box through a hole in the top. The terminus of the tubing was plugged and when inserted into the box came to rest *ca* 1 cm from the bottom, thereby permitting shrimp to pass freely beneath it. The test solution left the delivery device through two 1-mm dia. holes situated 5 mm from the tubing terminus. Flow rate was controlled with

an adjustable Buchler Polystaltic pump and a Gilmont No. 11 flowmeter.

Bioassays were standardized as follows. (1) Each test group of shrimp was left undisturbed beneath the covered frame for 60 min prior to testing. (2) The solution being tested was introduced into each compartment for a 2-min period at a flow rate of 2 ml per min. A single concentration of one solution was used for an entire test group. (3) Each positive response consisted of a shrimp moving to, and grasping, the delivery device within the 2-min period. Each shrimp was scored only once during the test. (4) Sea water alone was introduced for the control solution.

Preparation of extract and synthetic solutions

Aqueous extract of fresh blue crab (*Callinectes sapidus*) was prepared by stirring chopped muscle in five volumes of cold distilled water for 30 min followed by centrifugation at 12,000 *g* for 15 min at 4°C. The supernatant was decanted and frozen in small aliquots at −70°C.

Synthetic solutions were prepared in 3% NaCl. The pH was adjusted when necessary to 7 to 8 with 0.5N NaOH. Synthetic solutions were prepared such that the concentration of each individual substance was the same as measured in crab extract. Hence 1 μl of each synthetic solution contained the same amount of each indicated substance as measured in 1 μl of extract.

Homarine HCl was obtained from Aldrich. All other chemicals were from Sigma.

Chemical analyses

Analyses were conducted on the filtrate of a crab extract ultrafiltered at 4°C through an Amicon Centriflo CF50 membrane cone. Homarine, hypoxanthine and inosine were measured using reversed-phase high pressure liquid chromatography (Assenza and Brown, 1980) with a Waters Radial Pak column (C-18 bonded phase, 0.8 × 10 cm, 10-μm particle size). The initial eluant was 0.02 M KH_2PO_4, pH 5.7, and the final eluant was 3/2 methanol/water. A linear gradient of 0 to 23% final eluant was used over a 20-min period at a flow rate of 1.5 ml/min. Nucleotides were measured using a Waters SAX radial compression anion exchange column (0.8 × 10 cm, 10-μm particle size). Conditions were similar to those of McKeag and Brown (1978). Initial eluant was 0.007 M KH_2PO_4 plus 0.007 M KCl, pH 4.0, and final eluant was 0.25 M KH_2PO_4 plus 0.5 M KCl, pH 5.0. Flow rate was 5.0 ml/min. The solvent program started with 2 min of initial eluant, a 16-min concave gradient to the final eluant, and a hold with the final eluant for 12 min. Compound identification was based on retention time, co-chromatography with pure reference compounds, and, in the case of inosine and adenosine 5′-monophosphate, the u.v. absorption curves and absorbance ratios.

Lactic acid and trimethylamine oxide were identified and measured semiquantitatively on thin layer cellulose plates developed in a solvent system of butanol–acetic acid–water (6:2:2). Bromcresol green (Touchstone and Dobbins, 1978) and Dragendorff reagent (Bregoff *et al.*, 1953) were used for visualization. Concentrations of amino acids and betaine were taken from a prior analysis of an ultrafiltrate of a similar crab extract (Carr, 1978).

Statistical treatment of data

The stimulatory capacity of each solution was determined by recording the percent of the tested animals that gave a positive response over a range of at least five concentrations with a minimum of 36 animals (= 2 test groups) used at each concentration. Many of the data were analyzed by the procedure of Potency Probit Analysis (Daum and Givens, 1963) after transforming each response percentage to the percent of the maximum response obtained among the subsets of data being compared. This transformation defines the ED_{50} value as the effective dose for a half-maximal response. Regression lines ($\log_{10}$ dose vs probit response) shown in the text were drawn from these analyses.

RESULTS

The preliminary observations leading to this study were that aqueous extracts of crab and oyster were much more potent than appropriate synthetic mixtures of amino acids and betaine prepared as described earlier (Carr, 1978). Studies of the effects of feeding state and seasonality were undertaken to determine if major changes occurred in the relative activity of two stimulants. For these studies, one stimulant was an aqueous crab extract whereas the other was a synthetic mixture that contained 19 amino acids and betaine at the same concentrations as determined for each substance in a crab extract (Table 1, Group A Substances).

Effect of feeding state

Groups of shrimp with identical histories in the laboratory were deprived of food for 2, 3, 4, 5 and 6 days. During this time they were tested each day with crab extract and the synthetic mixture of amino acids plus betaine. Figure 1 shows that the extract was markedly more effective than the synthetic mixture. Also, the apparent effectiveness of both stimulants increased with the length of the starvation period. However, the crab extract continued to be the best stimulant, thereby showing that the differential responsiveness of the shrimp to the natural and the synthetic stimulants was not greatly affected by food deprivation. It is difficult to make meaningful comparisons between the rates of change in the apparent effectiveness of the two types of stimulant because of differences in the slopes of the regressions.

Effect of time of year

The stimulatory capacity of crab extract and the synthetic mixture of amino acids plus betaine was measured using shrimp collected each month from January to June 1982. Figure 2 shows that throughout this period the extract remained consistently more effective than the synthetic mixture. During this period, there was a close correlation between the direction and degree of shift in the dose–response curves for both types of stimulant. This close correlation indicated that real differences existed in the responsiveness of shrimp collected and tested at different times of the year. An examination of the dose–response curves for the crab extract suggests that shrimp collected in the late fall and winter months were the least responsive. ED_{50} values for crab extract tested with shrimp collected in November, January and February averaged 1.76 μl/ml (SEM ±0.895), whereas shrimp collected at other times had ED_{50} values that averaged 0.412 μl/ml (SEM ± 0.0981). It is not clear whether these differences in responsiveness were due to endogenous factors related to physiological state, and/or to an exogenous factor(s) such as water temperature. However, the period during which the shrimp were the least responsive (November through February) coincides with the period when the lowest water temperatures were encountered (see Fig. 2).

Measurement of additional substances in crab extract and assays of mixtures of greater complexity

Table 1 (Group B substances) shows the concentrations of the nucleotides, other purines, homarine,

Table 1. Concentrations of substances measured in extract of blue crab

Group A— Amino acids and betaine*		Group B— Additional substances	
Substance	Concentration (mM)	Substance	Concentration (mM)
Alanine†	2.14	Adenosine 5'-monophosphate‡ (AMP)	0.025
Arginine	2.29	Adenosine 5'-diphosphate‡ (ADP)	0.182
Asparagine	0.195	Adenosine 5'-triphosphate (ATP)	0.878
Aspartic acid	0.036	Hypoxanthine‡	0.033
Betaine‡	3.70	Inosine‡	0.069
Cysteine	0.120	Inosine 5'-monophosphate‡ (IMP)	0.158
Glutamic acid	0.284	Homarine*	0.469
Glutamine	2.95	Lactic acid†‡	4.35
Glycine‡	11.34	Trimethylamine oxide‡ (TMO)	3.38
Histidine	0.138		
Isoleucine	0.107		
Leucine	0.245		
Lysine	0.227		
Methionine	0.392		
Phenylalanine	0.093		
Proline	5.57		
Serine	0.265		
Taurine‡	1.88		
Threonine	0.404		
Tryptophan	0.131		
Tyrosine	0.101		
Valine	0.318		

*L-amino acids were used in synthetic solutions. Concentrations from Carr (1978).
†L(+) lactic acid used in synthetic solutions.
‡Substances present in sub-mixture cited in Fig. 3.

trimethylamine oxide and lactic acid measured in crab extract. The relative concentrations of the four identified nucleotides were as follows: ATP > ADP > IMP > AMP.

Figure 3 shows that mixtures containing the above substances plus the amino acids and betaine had a stimulatory capacity quite similar to that of the crab extract. These new mixtures were clearly more effective than the earlier one containing only the amino acids plus betaine. It was evident that at least some of these additional substances made a significant contribution to the activity of the mixture. Tests are currently underway to define the least complex mixture that accounts for most of the stimulatory capacity of the complete mixture of 31 substances. It is known that ATP is not required (see Fig. 3A). Further, a submixture containing only 12 of the substances is very effective yet yields a dose–response curve with a lesser

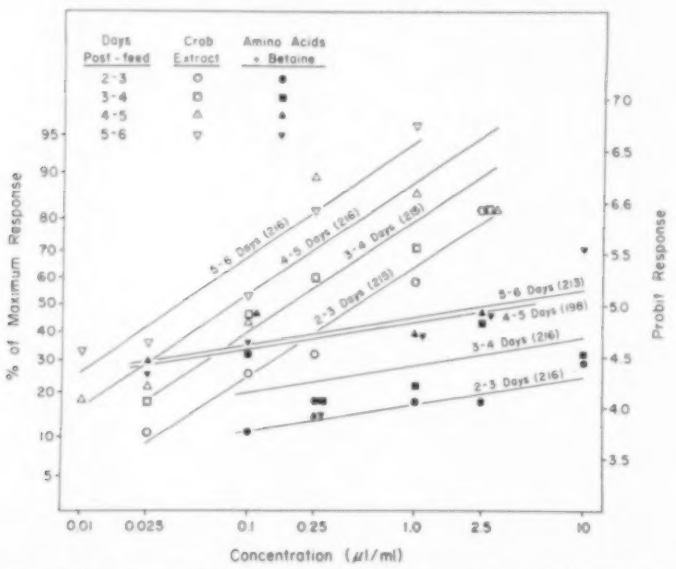

Fig. 1. Effect of feeding state on response of *P. pugio* to crab extract (open symbols) and mixture of amino acids plus betaine (closed symbols). Each data point is a mean response. Numbers in parentheses show number of shrimp tested. The effectiveness of both stimulants increased with the length of the starvation period, but the crab extract continued to be the best stimulant throughout the study.

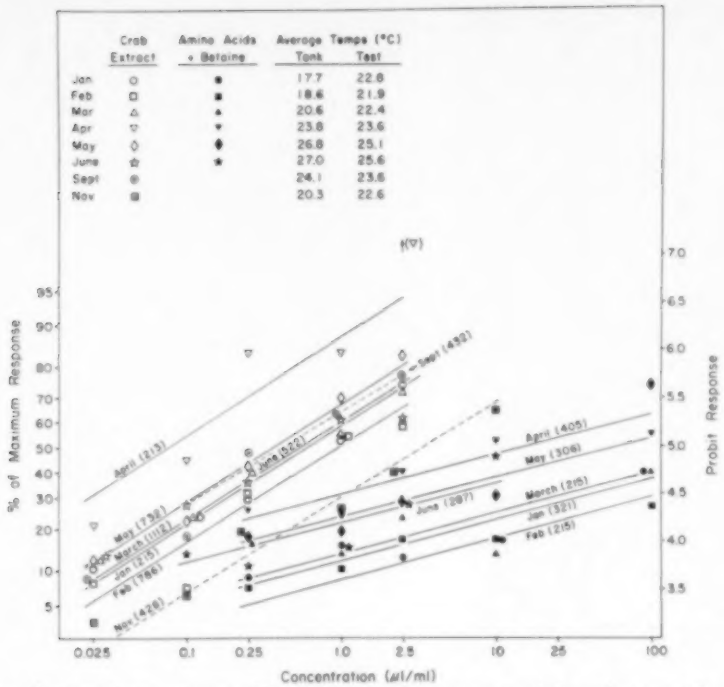

Fig. 2. Effect of seasonality on response of *P. pugio* to crab extract (open symbols) and mixture of amino acids plus betaine (closed symbols). Each data point is a mean response. Numbers in parentheses show number of shrimp tested. Water temperatures shown at top of figure were recorded in aquaria (Tank) and in bioassay boxes during the tests (Test). The effectiveness of both stimulants varied with the time of year (and/or temperature) but the crab extract remained more effective throughout. Only the extract was tested in September and November.

slope than the mixtures of greater complexity (see Fig. 3B).

The nucleotide, AMP, is the most potent single substance in the synthetic crab mixture (SCM). When tested alone, AMP stimulates a half-maximal response at a concentration of about 1 μM (Carr and Thompson, 1983). Figure 4 shows dose–response curves derived from assays of the SCM, AMP alone, and the amino acids plus betaine that are present in the SCM. When concentrations are expressed on a molar basis, AMP (ED_{30} *ca* 1.2 μM) is seen to be more potent than even the SCM (ED_{30} *ca* 6 μM). However, as

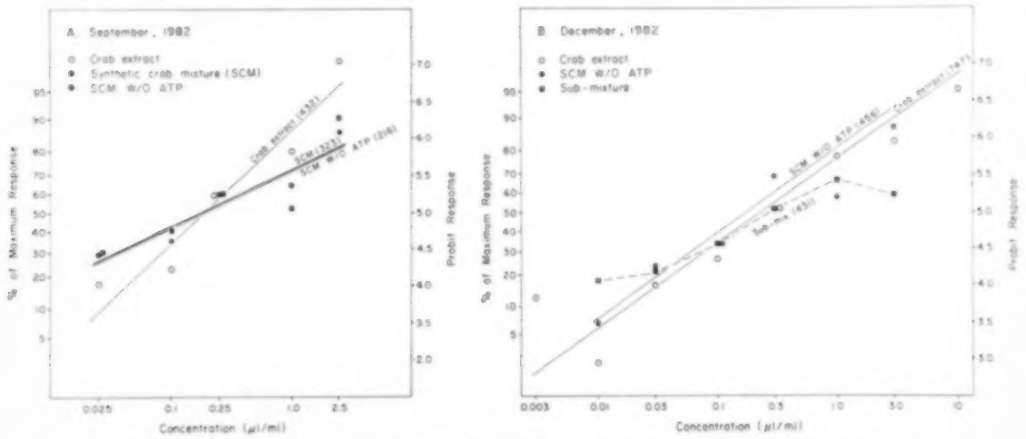

Fig. 3. Response of *P. pugio* to crab extract and synthetic crab mixtures containing amino acids, betaine and additional extract constituents shown in Table 1. Each data point is a mean response. Numbers in parentheses show numbers of shrimp tested. Because of diminished responsiveness, December shrimp (3B) were tested 4 days after feeding. September shrimp (3A) were tested 2 days after feeding (see Methods). The synthetic crab mixture (SCM, with or w/o ATP) had a stimulatory capacity quite similar to that of the crab extract. The sub-mixture in 3B contained the following 12 substances: alanine, betaine, glycine, taurine, AMP, ADP, IMP, hypoxanthine, inosine, homarine, lactic acid and TMO.

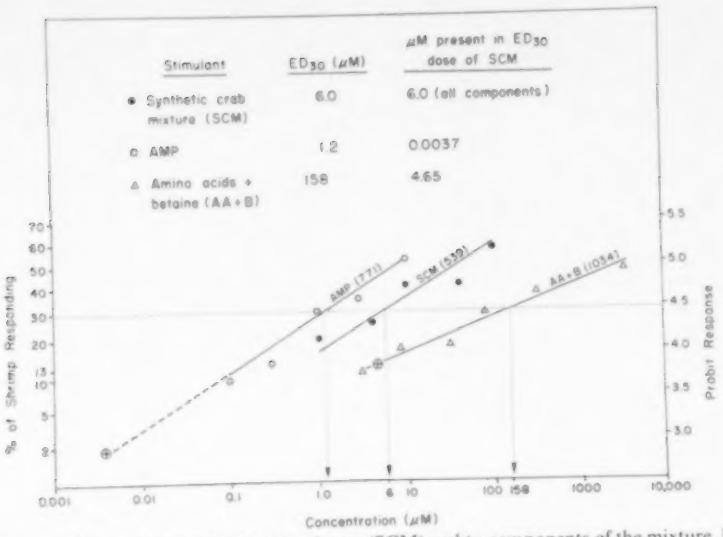

Fig. 4. Response of *P. pugio* to synthetic crab mixture (SCM) and to components of the mixture. Each data point is a mean response. Numbers in parentheses show number of shrimp tested. Regressions were drawn from results of Potency Probit Analysis of non-transformed data. Relevant computations are given above figure. On a molar basis, AMP is a more potent stimulant than SCM. However, the activity of SCM is much greater than predicted from the small amount of AMP it contains. Circled cross on curves for AMP and AA + B show site of extrapolation of predicted response values cited in text. Source of data as follows. SCM: Fig. 3, assays of Sept. 1982; AMP: Carr and Thompson (in press), assays of April through July 1982; AA + B: Fig. 2, assays of April through June 1982.

shown in the Discussion, the activity of the SCM is far greater than would be predicted from the small amount of AMP it contains.

DISCUSSION

In an earlier study, Mackie (1973) showed that a synthetic mixture of substances identified in a squid extract was highly attractive to the lobster, *Homarus gammarus*. This synthetic mixture contained amino acids, betaine, trimethylamine oxide, trimethylamine (TMA), homarine, hypoxanthine, inosine, adenosine 5′-monophosphate (AMP) and lactic acid. Except for TMA, this same group of substances comprised the ingredients of the synthetic crab mixture that was found in the current study to be so attractive to the shrimp, *P. pugio*. The results of this study clearly demonstrate that a natural crab extract, as well as a complex synthetic mixture containing the substances listed above, are each much more potent as chemoattractants for *P. pugio* than a synthetic mixture containing only amino acids and betaine. Although changes in the overall responsiveness of the shrimp to attractants were induced by feeding state and by time of year, these changes did not alter the relative effectiveness of the natural extract and a synthetic mixture of only the amino acids and betaine. However, the relative effectiveness of the extract and a synthetic mixture was markedly altered when the specific additional components described above were incorporated into the mixture.

The single most potent substance in the complete synthetic crab mixture (SCM) was AMP. As shown in Fig. 4, the ED_{30} of AMP was *ca* 1.2 μM whereas the ED_{30} of the SCM was *ca* 6 μM. However, the SCM is far more active than would be predicted on the basis of the AMP it contains. This is shown in Fig. 4 as follows. The concentration of AMP is an ED_{30} dose of the SCM is only about 0.004 μM. By extrapolation it can be seen that 0.004 μM AMP would be predicted to elicit a response from only about 2% of the population, whereas the actual response to the SCM is from about 30% of the population. Further, Fig. 4 shows that the concentration of amino acids plus betaine in an ED_{30} dose of the SCM elicits a response from only about 13% of the population. A summation of the predicted response to the AMP (2%) and to the amino acids plus betaine (13%) in an ED_{30} dose of the SCM is only about one-half of the actual response. Hence, a modest synergistic effect is indicated. This conclusion is supported by the fact that except for AMP, betaine, and the amino acid, glycine, none of the other substances present in the SCM have chemoattractant activity when tested alone at the concentrations at which they occur in an ED_{30} dose of the mixture. The actual contribution of these "other substances" to the activity of the SCM has yet to be resolved.

Recent assays of AMP analogs indicate that *P. pugio* possesses external chemoreceptors for this nucleotide that are quite similar in specificity to certain internal adenoside receptors of vertebrates (Carr and Thompson, in press). It is of interest that there are now several studies indicating that various animals possess external chemoreceptors for certain nucleotides. In certain hematophagous insects, the stimulus to gorge on blood is provided by adenosine nucleotides, especially ATP (Friend and Smith, 1977, 1982). Receptors responding preferentially to nucleotides, especially ADP, inosine 5′-monophosphate (IMP) and uridine 5′-monophosphate (UMP) are found on the lips of a fish, the puffer (Kiyohara *et al.*, 1975). In another fish, the turbot, IMP and the nucleoside,

inosine, have been identified as gustatory feeding stimulants (Mackie and Adron, 1978). In bovine taste papillae, certain nucleotides have been found to have a different sort of stimulatory effect on external chemoreceptors. In this case, the amount of glutamate bound to glutamate receptors is increased several fold by guanosine 5′-monophosphate, IMP and UMP (Torii and Cagan, 1980).

Studies of the effect of feeding state on responsiveness showed clearly that in *P. pugio* the apparent effectiveness of both a natural and a synthetic mixture increased with the length of the starvation period (see Fig. 1). Increases in behavioral responsiveness to chemical stimulants have been reported to accompany food deprivation in two other crustaceans, the lobster, *H. gammarus* (Mackie and Shelton, 1972), and the blue crab, *Callinectes sapidus* (Pearson and Olla, 1977). In insects, food deprivation has been found to cause an increase in chemoreceptor sensitivity that may be induced by hormones (Bernays and Chapman, 1972; Bernays and Mordue, 1973). Similar studies of this aspect of sensory modulation are unknown in crustaceans. The reader should see Ache (1982) for a general review of other aspects of chemoreception in Crustacea.

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CHANGES IN BIOCHEMICAL COMPOSITION IN THE RED GROUPER, *EPINEPHELUS AKAARA* (TEMMINCK AND SCHLEGEL), AND THE BLACK SEA BREAM, *MYLIO MACROCEPHALUS* (BASILEWSKY), DURING HYPOXIC EXPOSURE

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Abstract—1. Red groupers and black sea breams exposed to 4 and 2.5 mg O_2 l^{-1} for 1–7 hr showed no increase in serum and tissue lactate concentration and only slight changes in other tissue metabolites and electrolytes.

2. In both species subjected to 1 mg O_2 l^{-1}, there was marked hyperglycaemia, hyperlactaemia, hepatic glycogenolysis and elevation of hepatic lactate content, but the lactate and glycogen contents of the muscles were unaffected.

3. Both species can obtain enough oxygen to prevent anaerobiosis at 4 to 2.5 mg O_2 l^{-1} and fermentation of hepatic glycogen to lactate is the principal pathway of energy production in 1 mg O_2 l^{-1}.

4. In both species, serum Na^+, K^+ and Ca^{2+} concentrations were unchanged after exposure to 4 to 2.5 mg O_2 l^{-1} but all three parameters were elevated after exposure to 1 mg O_2 l^{-1}, suggesting that osmoregulatory failure could occur in extremely hypoxic conditions.

5. The black sea bream, but not the red grouper, accumulated lipids in the liver in addition to lactate when exposed to all levels of hypoxia.

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INTRODUCTION

Some teleosts are known to be able to withstand considerable periods of environmental hypoxia (e.g. Blazka, 1958; Van den Thillart and Kesbeke, 1978). While extensive investigations have been carried out to determine the various cardiovascular and respiratory adjustments to hypoxia (Randall, 1970a, b), it is also recognized that different tissues of fish can sustain varying levels of anaerobic metabolism. However, it is not possible to generalize on a common adaptive strategy of anaerobiosis because contrasting patterns of anaerobic metabolism has been reported in different species. In most teleosts, fermentation of glucose or glycogen to lactate provides the main source of energy production in hypoxic conditions (Heath and Pritchard, 1965; Bandurski *et al.*, 1968; Wittenberger, 1968; Hunn, 1969; Burton, 1970a, b; Burton and Spehar, 1971; Van den Thillart *et al.*, 1976; Burggren and Cameron, 1980; Jørgensen and Mustafa, 1980). However, some species were found to possess pathways which depart from this general scheme. Blazka (1958) has shown that anaerobic metabolism in the carp, *Carassius carassius*, did not result in the accumulation of lactic acid. Instead, short-chain fatty acids accumulated and CO_2 was excreted. In contrast, Johnston (1975), working on the same species, showed that in addition to the accumulation of lactate, alanine and succinate were also formed in the red muscles. Similar findings were also reported for *Torpedo marmorata* exposed to hypoxia (Hughes and Johnston, 1978). In *Platichthys flesus*, lactate, alanine and succinate were all observed to accumulate after hypoxic exposure but the amount of these end products accumulating in different tissues was different (Jørgensen and Mustafa, 1980). It is therefore likely that tolerance to hypoxia in different species can be related to a fundamental difference in anaerobic metabolic machinery.

This problem, however, is complicated by two factors. Firstly, different tissues have different powers of sustaining anaerobic metabolism. For example, the neural tissues generally suffer irreversible damage when subjected to oxygen depletion, which is in contrast to the extreme tolerance of oxygen deprivation in the white muscle (Wittenberger and Diaciuc, 1965). However, the brain of some vertebrates is capable of prolonged anaerobiosis (Belkin, 1968). The second complicating factor is the branchial apparatus. It is well known that the fish gill plays an important role in osmoregulation (Conte, 1969) in addition to its respiratory function. Hypoxic conditions which lead to respiratory changes may therefore result in alterations in body electrolyte balance.

This study reports on the effects of hypoxia on serum glucose, lactate and electrolyte levels and concentrations of glycogen, lactate and lipid in the liver, brain and muscle of the red grouper *Epinephelus akaara* and the black sea bream *Mylio macrocephalus*. In a previous study (Wu and Woo, 1983), we have found that *M. macrocephalus* has a greater ability to withstand hypoxia than *E. akaara*, and such disparity has been partially ascribed to a greater affinity for oxygen in the blood of *M. macrocephalus*. The present study attempts to provide further explanation for such a disparity between these two species at the biochemical level.

MATERIALS AND METHODS

Experimental animals

Red groupers (*Epinephelus akaara*) (281 ± 19 g, $\bar{X}$ ± SEM) and black sea breams (*Mylio macrocephalus*) (254 ± 14 g, $\bar{X}$ ± SEM) were obtained from a sea-pen (28°C, 30‰) in August 1981. Fish were transported to the laboratory and acclimated in well-aerated seawater (25 ± 0.5°C, 30‰) for 48 hr before experimentation. Fish were not fed during the entire acclimation period.

Hypoxic exposure

As previously described by Wu and Woo (1983), a continuous flow system was set up to provide a desired, constant level of ambient dissolved oxygen level throughout the entire experimental period. Essentially, the system consisted of a seawater reservoir connected to eight plexiglas experimental tanks. Seawater (30‰) was pumped from the reservoir to the experimental tanks. Red groupers and black sea breams were acclimated in the experimental tanks for 24 hr prior to experiments. Fully aerated seawater (8.0–8.5 mg O_2 l^{-1}) was provided during acclimation. At the start of the experiment, the level of dissolved oxygen in the system was adjusted to the desired level (±0.1 mg O_2 l^{-1}) by bubbling pure nitrogen/oxygen into the reservoir and the experimental tanks through a series of air stones. The time required to bring the dissolved oxygen level in the system down from 8 mg O_2 l^{-1} to 4 and 1 mg O_2 l^{-1} was about 5 and 15 min respectively. Three different levels of hypoxia (4, 2.5 and 1 mg O_2 l^{-1}) were tested in the present study. Fish were sampled at fixed time intervals after the desired hypoxic level was reached.

Fish sampling

Seven fish of each species were sampled from the 7 mg O_2 l^{-1} treatment and served as controls. Seven fish were sampled at random at 1, 3, 5 and 7 hr intervals from the 4 and 2.5 mg O_2 l^{-1} treatments. For the 1 mg O_2 l^{-1} treatment, the red groupers were sampled at 0.5, 1, 2 and 4 hr intervals, and the black sea bream at 1, 2, 3 and 7 hr intervals instead.

Fish were rapidly removed from the respirometers and stunned by a blow on the head. Blood was collected into a syringe by cardiocentesis. Whole blood was allowed to clot at room temperature for 30 min and centrifuged to obtain serum. The liver, brain and a piece of epaxial muscle were rapidly removed. The entire sampling procedure took less than 4 min. All samples were immediately frozen at −20°C until analysis.

Metabolite and electrolyte analyses

Serum Na^+, K^+ and Ca^{2+} concentrations were determined using flame photometry (ISA Biologie) after appropriate dilution of the serum samples. Serum glucose was determined using a coupled glucose oxidase–peroxidase reaction according to Sigma Procedure (No. 510). Serum lactate level was estimated using lactate dehydrogenase according to Hohorst (1962).

Tissue samples were homogenized in distilled water (1 : 10; w/v) using an Ultra-Turrax Homogenizer. The whole homogenate was used to determine tissue glycogen concentrations (Murat and Serfaty, 1974). For the determination of tissue lactate content, tissues were homogenized in 1 M perchloric acid and the supernatant was used for the determinations. Tissue samples were dried to constant weight at 80°C and then the lipids were extracted into petroleum either (b.p. 60–80°C). The defatted samples were weighed and the percentages of water and lipid in the tissues were calculated using weight differences.

Statistical analysis

Data are presented as means ± SEM. Statistical difference between all the treatment groups were assessed using Duncan's New Multiple Range Test.

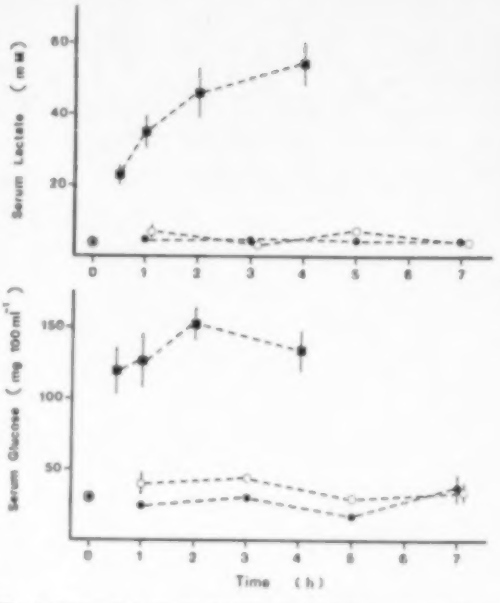

Fig. 1. Changes in serum glucose and lactate concentrations in *Epinephelus akaara* exposed to different levels of hypoxia. ⊛, 7 mg O_2 l^{-1}; ●, 4 mg O_2 l^{-1}; ○, 2.5 mg O_2 l^{-1}; ■, 1 mg O_2 l^{-1}.

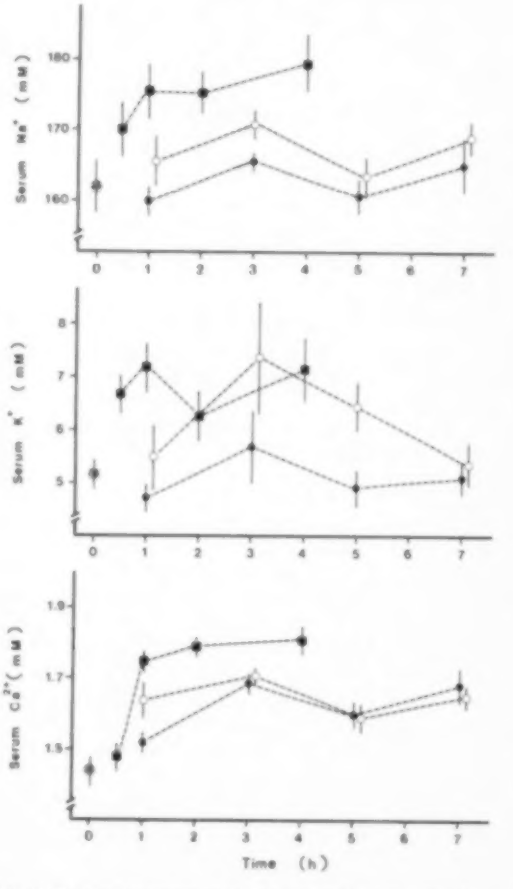

Fig. 2. Changes in serum Na^+, K^+ and Ca^{2+} concentrations in *Epinephelus akaara* exposed to different levels of hypoxia.

RESULTS

Epinephelus akaara (*Figs* 1–5)

Both serum glucose and lactate levels of fish kept at 4 and 2.5 mg O_2 l^{-1} from 1 to 7 hr were not significantly different from the normoxic values but were markedly elevated when exposed to 1 mg O_2 l^{-1} for 0.5–4 hr (Fig. 1). Serum glucose was elevated by 308% ($P < 0.001$) after 0.5 hr exposure to 1 mg O_2 l^{-1} (Fig. 1). There was a trend of increasing hyperglycaemia as fish were kept for longer periods in 1 mg O_2 l^{-1}, reaching a peak (420% of the normoxic value, $P < 0.001$) after 2 hr of exposure. High serum glucose level was maintained until the end of the experiment. Serum lactate showed a progressive increase from 0.5–4 hr after exposure to 1 mg O_2 l^{-1} (0.5 hr: +452%; 1 hr: +754%; 2 hr: +1012%; 4 hr: +1211%) (Fig. 1). The values for serum Na^+, K^+ and Ca^{2+} were also unchanged after exposure to 4 and 2.5 mg O_2 l^{-1} but increases in all three variables became apparent as the ambient oxygen level was further lowered to 1 mg O_2 l^{-1} (Fig. 2). Statistically significant increases in serum Na^+ (+8.3%; $P < 0.05$) and Ca^{2+} (+21%; $P < 0.001$) concentrations were apparent after exposure to 1 mg O_2 l^{-1} for 1 hr. High levels of serum Na^+ (+10.7%; $P < 0.02$) and Ca^{2+} (+25.7%; $P < 0.001$) were maintained until the end of the experiment. Serum K^+ level also increased after exposure to 1 mg O_2 l^{-1}, although the increase at 2 hr was only marginally significant (0.5 hr: +29.4%; 1 hr: +38.9%; 4 hr: +38.1%) (Fig. 2).

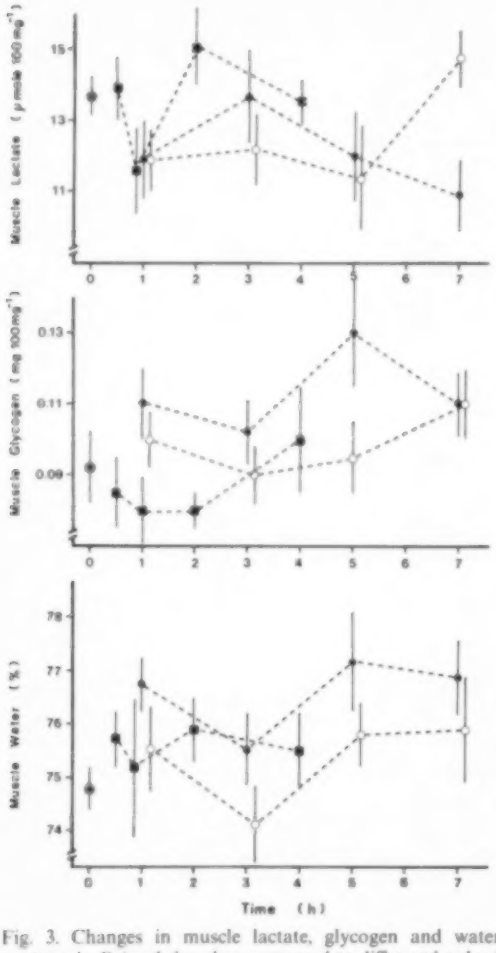

Fig. 3. Changes in muscle lactate, glycogen and water contents in *Epinephelus akaara* exposed to different levels of hypoxia.

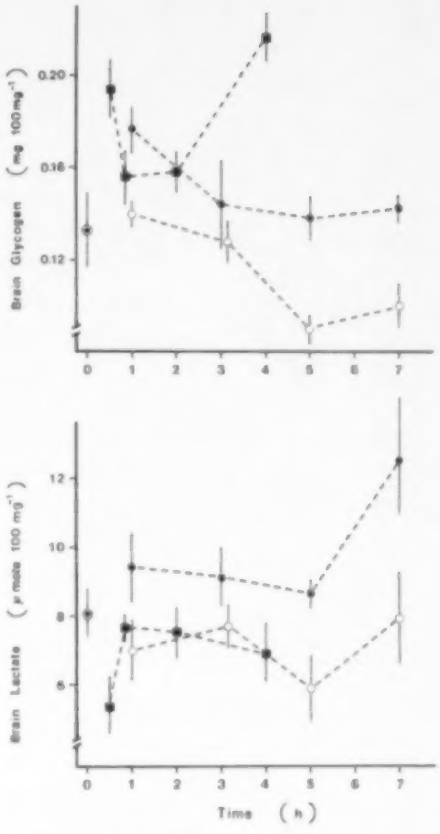

Fig. 4. Changes in brain lactate and glycogen contents in *Epinephelus akaara* exposed to different levels of hypoxia.

Both muscle lactate and glycogen concentrations were unaffected throughout 7 hr of hypoxic exposure (Fig. 3). Lactate concentration in the brain tissue was quite variable after hypoxic exposure and no clear trend of change could be discerned (Fig. 4). Brain glycogen content was also unaffected upon exposure to 4 and 2.5 mg O_2 l^{-1} but there were significant transient increases after exposure to 1 mg O_2 l^{-1} for 0.5 hr (+45%; $P < 0.02$) and 4 hr (+62%; $P < 0.001$) (Fig. 4). There was an overall trend of decrease in liver glycogen content after hypoxic exposure but statistical significance could only be observed in the groups exposed to 2.5 mg O_2 l^{-1} for 5 hr (−45%; $P < 0.05$) and 1 mg O_2 l^{-1} for 1 hr (−52%; $P < 0.02$), due to large variances in this parameter (Fig. 5). Change in liver lactate was biphasic: there was a significant decrease in the variable after 1–7 hr at 4 mg O_2 l^{-1} (maximum decrease at 3 hr: 43.6%; $P < 0.001$) (Fig. 5). However, liver lactate concentration approached the normoxic value throughout the entire period in 2.5 mg O_2 l^{-1} and during 0.5–1 hr in 1 mg O_2 l^{-1}. However, the parameter was markedly elevated at 2 (+44.6%;

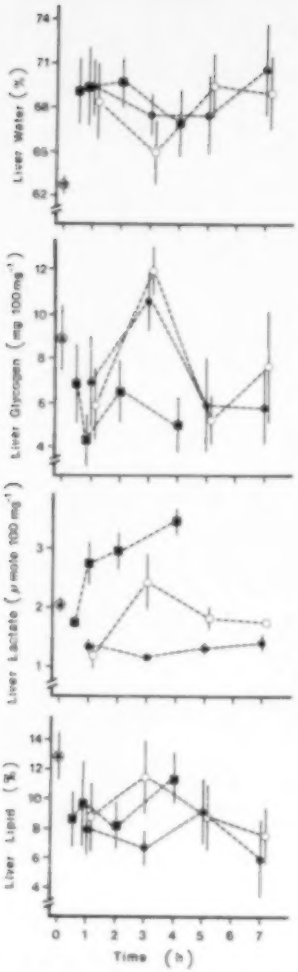

Fig. 5. Changes in liver water, glycogen, lactate and lipid contents in *Epinephelus akaara* exposed to different levels of hypoxia.

$P < 0.02$) and 4 hr (+70%; $P < 0.001$) in 1 mg O_2 l^{-1} (Fig. 5).

There was a general trend of increasing liver water in fish subjected to hypoxia (Fig. 5). Liver water was significantly higher in fish subjected to 4 mg O_2 l^{-1} for 1, 3 and 7 hr, to 2.5 mg O_2 l^{-1} for 5 and 7 hr and to 1 mg O_2 l^{-1} for 0.5–2 hr (Fig. 5) but no changes could be observed in muscle water (Fig. 3). The concentration of total lipids in the liver, however, was unaffected by all levels of hypoxic exposure (Fig. 5).

Mylio macrocephalus (*Figs* 6–10)

All the measured serum variables were unchanged throughout the entire period of exposure to 4 and 2.5 mg O_2 l^{-1} (Figs 6 and 7). Serum glucose, however, was elevated by 88.7% ($P < 0.001$) and 69.4% ($P < 0.05$) after exposure to 1 mg O_2 l^{-1} for 1 and 2 hr respectively. Serum glucose subsequently fell to near normoxic level by 7 hr in 1 mg O_2 l^{-1} (Fig. 6). Serum Na^+ was increased from 2 to 7 hr in 1 mg O_2 l^{-1} (2 hr: +19%, $P < 0.05$; 3 hr: +9.2%, $P < 0.001$; 7 hr: +16%, $P < 0.001$) (Fig. 7). Serum Ca^{2+} remained unchanged after exposure to 1 mg O_2 l^{-1} for 1–2 hr,

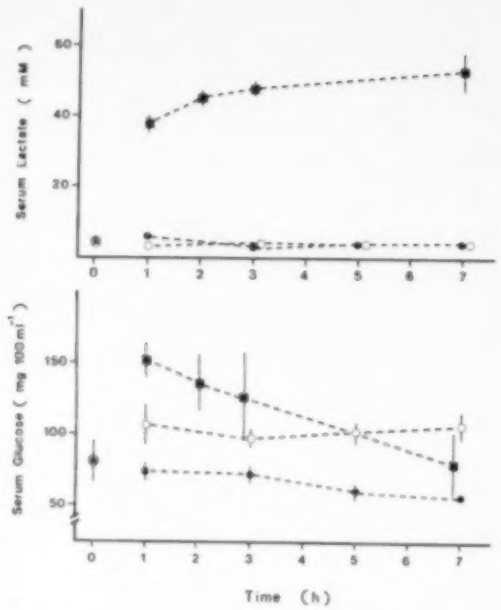

Fig. 6. Changes in serum glucose and lactate concentrations in *Mylio macrocephalus* exposed to different levels of hypoxia.

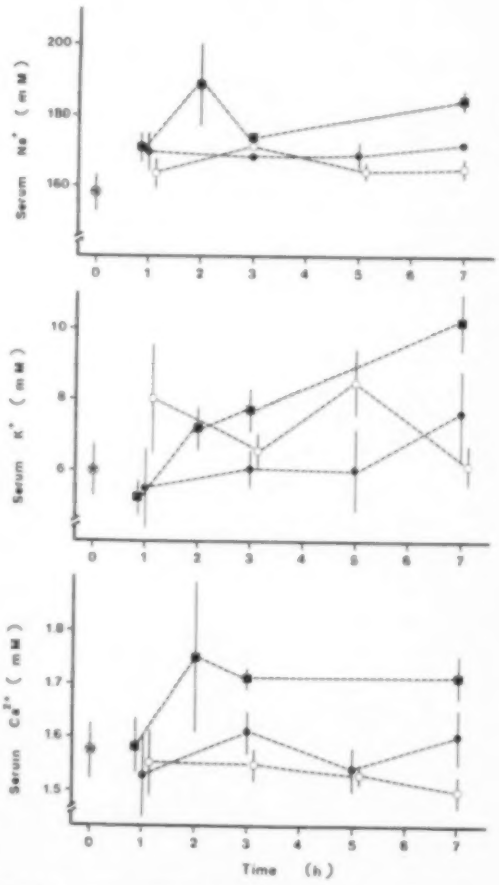

Fig. 7. Changes in serum Na^+, K^+ and Ca^{2+} concentrations in *Mylio macrocephalus* exposed to different levels of hypoxia.

but was elevated from 3 to 7 hr (both +8.9%; $P < 0.05$) (Fig. 7). Serum K^+ also remained unchanged from 1 to 3 hr in 1 mg O_2 l^{-1}, but was elevated after 7 hr (+69%, $P < 0.01$) (Fig. 7). Marked elevation of serum lactate was observed in fish exposed to 1 mg O_2 l^{-1} for 1–7 hr (1 hr: +831%; 2 hr: +1019%; 3 hr: +1093%; 7 hr: +1223%) (Fig. 6).

There were no changes in the glycogen and lactate contents of both muscle (Fig. 8) and brain (Fig. 9) throughout the entire period of exposure to all three levels of hypoxia. Liver lactate content was unchanged in 4 and 2.5 mg O_2 l^{-1} but was markedly elevated after exposure to 1 mg O_2 l^{-1} for 1–7 hr (maximum increase at 1 hr: +94.1%, $P < 0.001$) (Fig. 10). Liver glycogen level gradually fell in fish subjected to 4 mg O_2 l^{-1}, and was significantly below normoxic value by as much as 62.8% ($P < 0.01$), 56.6% ($P < 0.02$) and 54.2% ($P < 0.02$) at 3, 5 and 7 hr respectively (Fig. 10). However, the liver glycogen contents of fish after exposure to 2.5 mg O_2 l^{-1} for 1–7 hr were all not statistically different from the normoxic value, but were further decreased in fish subjected to 1 mg O_2 l^{-1} for 2 (−62.5%, $P < 0.01$), 3 (−68.2%, $P < 0.02$) and 7 hr (−72.6%, $P < 0.01$) (Fig. 10).

Liver water was not affected by exposure to 4 mg O_2 l^{-1} (Fig. 10). However, liver water was significantly

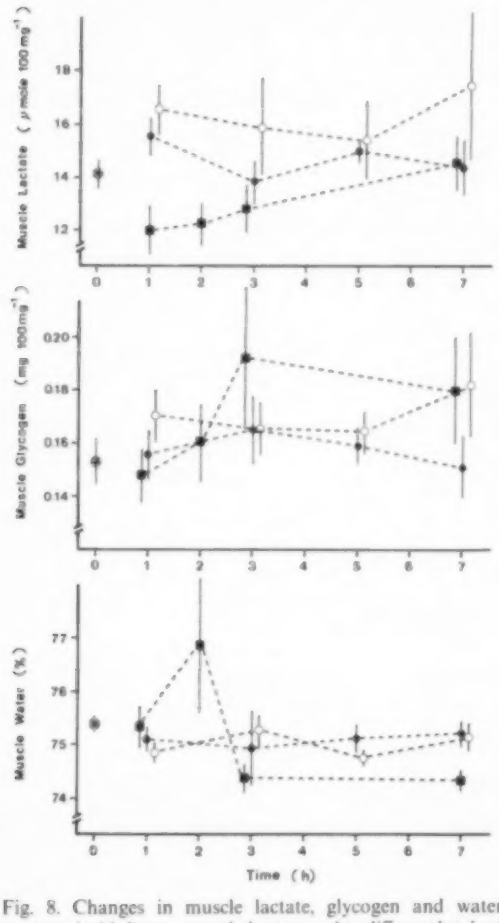

Fig. 8. Changes in muscle lactate, glycogen and water contents in *Mylio macrocephalus* exposed to different levels of hypoxia.

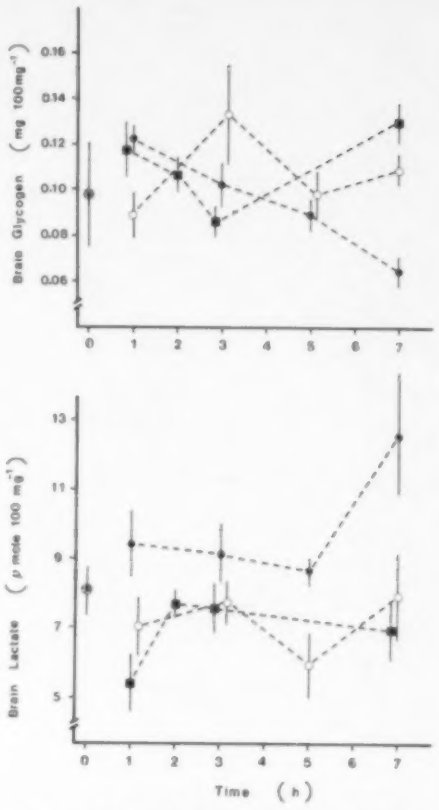

Fig. 9. Changes in brain lactate and glycogen congents in *Mylio macrocephalus* exposed to different levels of hypoxia.

reduced after exposure to 2.5 mg O_2 l^{-1} for 1–7 hr and to 1 mg O_2 l^{-1} for 3–7 hr (Fig. 10). There was a trend of lipid accumulation in the liver of fish subjected to all levels of hypoxia (Fig. 10). All the values of liver lipid in fish subjected to hypoxia were significantly higher than the normoxic value except for the group kept at 4 mg O_2 l^{-1} for 7 hr. A maximum increase of 220% in liver lipid content was observed in fish subjected to 1 mg O_2 l^{-1} for 3 hr (Fig. 10). Muscle water was, however, unaffected by exposure to all levels of hypoxia (Fig. 8).

DISCUSSION

In our previous study (Wu and Woo, 1983), it has been demonstrated that both the black sea bream and the red grouper possessed considerable powers of hypoxic tolerance. No mortality or abnormal behaviour was observed for either species in 2 mg O_2 l^{-1} for up to 7 hr (Wu and Woo, 1983) despite venous P_{O_2} being markedly decreased. The present study extends our previous findings to the biochemical level: in both species kept at 4 and 2.5 mg O_2 l^{-1} for 1–7 hr, only slight changes in tissue metabolite and electrolyte levels could be discerned. This suggests that both species are able to maintain homeostasis down to 2.5 mg O_2 l^{-1}. It must be emphasized that in both species kept at 4–2.5 mg O_2 l^{-1}, no signs of increased anaerobiosis (lactate production) was evident. This would indicate that during moderate hypoxia (4–2.5 mg O_2 l^{-1}), supply of oxygen to the tissues

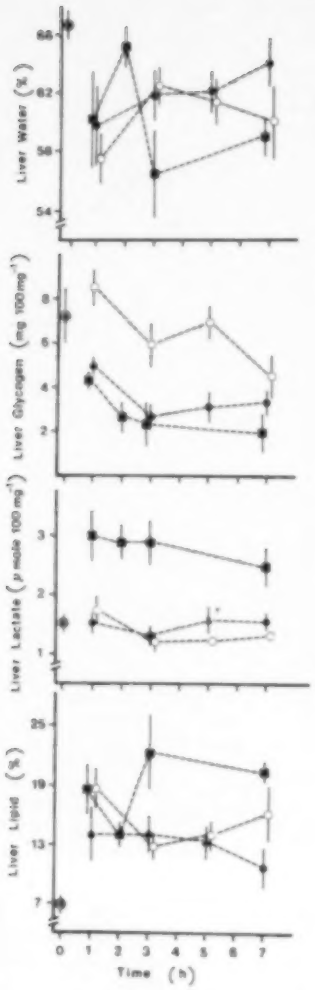

Fig. 10. Changes in liver water, glycogen, lactate and lipid contents in *Mylio macrocephalus* exposed to different levels of hypoxia.

would be adequate to prevent anaerobiosis: a similar observation was also made by Soivio *et al.* (1981) on *Salmo gairdneri*. These findings, together with our earlier observations that both the red grouper and the black sea bream reduce their oxygen consumption as well as opercular beating rates during hypoxia (Wu and Woo, 1983), would indicate that the fundamental adaptive strategy in both species is to conserve oxygen at moderate hypoxic levels, rather than to hyperventilate to increase oxygen uptake, as reported in other species (Randall, 1970b).

However, as the ambient oxygen level is further reduced, such conservatory mechanisms must fail and the fish would eventually resort to anaerobic respiration. Indeed, signs of anaerobiosis were evident in both species exposed to 1 mg O_2 l^{-1}. There were marked hyperglycaemia, hyperlactaemia, hepatic glycogenolysis and concomitant elevation of hepatic lactate content. Thus the classical pathway of fermentation of glycogen to lactate to provide energy in hypoxic conditions appears to be operative in both species at 1 mg O_2 l^{-1}. Although such a pathway has been well established, the site of glycogen mobilization is highly variable among teleost species. In the cutthroat trout (*Salmo clarki*), blue gill sunfish (*Lepomis macrochirus*) (Heath and Pritchard, 1965) and the flounder (*Platichthys flesus*) (Jørgensen and Mustafa, 1980), both liver and muscle glycogen were used as the anaerobic substrates. In the carp (*Carassius carassius*), muscle glycogen is used instead of liver glycogen (Johnston, 1975). In both the black sea bream and the red grouper, the site of glycogen mobilization appears to reside only in the liver. The lactate and glycogen content of the muscles of both species were unaffected. Lactate was also observed to accumulate in the muscle of *Periophthalmus australis* (Bandurski *et al.*, 1968), *Ictalurus nebulosus* (Burton, 1970a) and *L. macrochirus* during hypoxia (Burton, 1970b), suggesting muscle glycogenolysis. However, the fate of muscle lactate in fish is uncertain. In mammals, lactate is an important gluconeogenic substrate in the liver and kidney. Lactate derived from skeletal muscles is transported to the liver and glucose is then re-synthesized from lactate. Hepatic gluconeogenesis from lactate in teleosts has seldom been investigated. Some authors have provided evidence that the teleost liver has a negligible role in metabolizing lactate (Black *et al.*, 1966) and that lactate uptake by the teleost liver is minimal (Dando, 1969). In both the black sea bream and red grouper, however, there is no evidence for muscle glycogenolysis and lactate production. Lactate accumulation in the liver at 1 mg O_2 l^{-1} is probably the result of hepatic fermentation of glycogen to lactate and accumulation due to an inadequate diffusion of the metabolite into the bloodstream, suggesting deterioration of hepatic circulation during hypoxia (Dando, 1969).

It is assumed in the previous discussion that lactate production corresponds quantitatively to glycogen utilization. However, the possibility exists that end products of anaerobic metabolism other than lactate may be formed. Blazka (1958) observed the accumulation of short-chain fatty acids instead of lactate during hypoxia in the carp. Subsequently, many authors could not find the accumulation of lipids (e.g. Burton and Spehar, 1971) but noted the formation of alanine and succinate (Johnston, 1975; Hughes and Johnston, 1978; Jørgensen and Mustafa, 1980) in addition to lactate. The goldfish was observed to produce NH_3 and oxidize glycogen to CO_2 in the total absence of oxygen (Van den Thillart and Kesbeke, 1978). The results of the present study, however, tend to agree with Blazka's (1958) findings in carps. The total lipids in the liver of the black sea bream during hypoxia was markedly higher than the normoxic controls. Furthermore, the accumulation of liver lipids in the black sea bream was directly dependent on the degree of hypoxia: more lipids accumulated under lower ambient oxygen levels. It is important to note that liver lipid content of the black sea bream was elevated even at ambient levels of oxygen which did not result in increased lactate production (4–2.5 mg O_2 l^{-1}). It therefore seems that the general metabolic response of the black sea bream depends on the severity of hypoxia: lipids were produced at moderate levels of hypoxia and lactate was formed additionally at extremely low ambient oxygen tensions. No accumulation of lipid was found in the red grouper during hypoxia, indicating that accumulation of lipids

during hypoxia in fish may only have a sporadic occurrence.

In both the red grouper and the black sea bream, serum Na^+, K^+ and Ca^{2+} concentrations were unchanged after exposure to 4–2.5 mg O_2 l^{-1} but all three parameters were markedly elevated after esposure to 1 mg O_2 l^{-1}. Elevated serum electrolytes levels is indicative of osmoregulatory imbalance arising from (i) haemoconcentration due to the loss of water from the vascular compartment and/or (ii) failure to excrete the constant influx of salts from seawater either at the gills or kidneys, leading to the retention of electrolytes in the blood. Haemoconcentration, however, was unlikely because the different ions (and other blood metabolites) were not elevated to the same extent and there was no evidence of change in muscle hydration. Therefore, the elevation of serum electrolytes can be attributed to impairment of either branchial or renal function, or a combination of both.

Dysfunction of osmoregulatory organs is probably the result of an inadequate supply of energy to maintain the normal function of these organs during hypoxia. Since fish hypoosmoregulate in seawater, one would expect osmoregulatory failure to lead to the elevation of serum ions in a seawater fish and the opposite would occur in a freshwater fish. Our present findings are in agreement with these theoretical predictions. In *Tilapia nilotica* subjected to exhausting exercise, an increase in plasma osmolarity was observed in seawater but a decrease was noted in fresh water (Farmer and Beamish, 1969), supporting the above notion. However, significant elevation of plasma (Kirk, 1974) and urine (Hunn, 1969) electrolytes has also been observed in freshwater teleosts subjected to hypoxia. It would seem, therefore, that the extent of osmotic/ionic disturbance during hypoxic exposure could depend on some other factors in addition to salinity. Ishioka (1982) only noted transient hypernatremia in red sea breams subjected to rapid decrease in oxygen content. Soivio *et al.* (1981) were unable to detect any changes in serum electrolytes in rainbow trouts subjected to moderate hypoxia. In our present experiments with the black sea bream and the red grouper, electrolyte imbalance was not observed until extreme hypoxia (1 mg O_2 l^{-1}) was reached. The extent of osmoregulatory imbalance during hypoxia therefore seems to depend on the severity and duration of hypoxia as well as the prevailing salinity.

One of the aims of our present experiments is to provide a biochemical explanation for a better hypoxic tolerance in the black sea bream. In most vertebrates, brain tissue is highly susceptible to damage by hypoxic exposure but some reptilian (Belkin, 1968) and teleost (Congleton, 1974) brains are capable of extended anaerobiosis. In the present study, brain lactate concentration of both species did not increase and brain glycogen content was not depleted even at 1 mg O_2 l^{-1}, indicating that the brain may obtain substrates from the blood stream in both species. A continuous exchange of metabolites (glucose and lactate) between circulating blood and brain is presumed to be vital for anaerobic survival (Congleton, 1974).

Another biochemical basis for different degree of hypoxic tolerance has been ascribed to the amount of liver glycogen storage (Congleton, 1974; Johnston, 1975). This is to be expected since in hypoxia, glycogen is the sole source of energy production. In species such as the carp which exhibits pronounced hypoxic tolerance, liver glycogen content as high as 22 g $100g^{-1}$ liver has been reported (Johnston, 1975). Furthermore, freshwater teleosts, which are usually more tolerant to hypoxia than marine teleosts, generally have a much higher liver glycogen storage (Demael-Suard *et al.*, 1974; Johnston, 1975; Jørgensen and Mustafa, 1980; Woo and Murat, 1981). However, this does not seem to be related to the different degree of hypoxic tolerance exhibited by the black sea bream and red grouper, since both species have similar liver glycogen storage and both can draw upon liver glycogen stores as the anaerobic substrate.

The only significant biochemical difference between the red grouper and the black sea bream identified in this study is that the latter can synthesize lipids in the liver during hypoxia. Whether this phenomenon is related to a higher hypoxic tolerance in the black sea bream is uncertain. However, it is interesting to note that the crucian carp, which can survive in strictly anaerobic conditions for 5½ months, has the ability to synthesize lipids in anoxic conditions (Blazka, 1958). Further experiments are required to clarity this point and these are being actively pursued in our laboratories at the present time.

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ENDOTOXIN AND PROSTAGLANDIN E_1 FAIL TO INDUCE FEVER IN A TELEOST FISH*

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Abstract—1. Pumpkinseed sunfish (*Lepomis gibbosus*) developed no behavioural fever (increase in preferred temperature) when injected with *E. coli*-endotoxin or prostaglandin E_1 (PGE_1).

2. After intracranial injection of PGE_1 the animals were sedated and consecutively hypo- or hyperthermia-like effects which mimicked fever, occurred in shuttleboxes of the type used.

3. Previous results on fever induction in ectotherms have to be reconsidered critically.

INTRODUCTION

Fever is commonly described as an elevated thermoregulatory "set-point". While endothermic vertebrates raise their body temperature by autonomical and behavioural means, ectothermic species depend largely on behaviour, e.g. selecting appropriate temperatures in their environment.

It is well known that as well as bacterial pyrogens, prostaglandins of the E-class can elicit fever in homeotherms (Milton, 1982). Following applications of such pyrogens behavioural fever-like phenomena (increase in preferred temperature) have also been described for some ectotherms such as fishes, amphibians, reptiles and arthropods (for review see Reynolds and Casterlin, 1982; Casterlin and Reynolds, 1982). Since these results are very scarce and partly contradictory, the aim of the present work was to compare the influence of *E. coli*-endotoxin and prostaglandin E_1 (PGE_1) on temperature preference in a single species (*Lepomis gibbosus* L.) by means of identical methods. Because thermoregulation in small fish is largely behavioural, special care was taken to characterize the motility of the fish following pyrogen application.

MATERIALS AND METHODS

Twenty-eight pumpkinseed sunfish (*Lepomis gibbosus*, 17.56 ± 2.77 g body weight) were allowed to thermoregulate individually in an electronic shuttlebox (Fig. 1). The functional principle of the shuttlebox was derived from Neill *et al.* (1972) and Reynolds (1977). The fish were able to control water temperatures by swimming back and forth between two chambers of different water temperatures. The two chambers differed in temperature by 2–3.5°C so that a small spatial gradient was always present. By passing over a barrier between the chambers and sequentially interrupting two light beams monitored by phototransistor relays, the fish initiates heating or cooling respectively. If the temperature of the respective chamber reaches the upper or lower avoidance temperature of the fish, the fish is able to move along the spatial gradient to the other chamber, which is nearer to the preferred temperature. The number of passages between chambers was used as a measure of locomotor activity.

Following a 24-hr adjustment period baseline data (final thermal preference = mean hourly occupied temperatures, and swimming activity = lightbeam interruptions) were collected for another 24 hr period.

In the morning (8.00–8.30 a.m.) of the third day the fish were injected with one of the following:

(a) *E. coli*-endotoxin, serotype 055:B5 (Sigma, Munich), i.p., 100 μg/kg body wt;
(b) sterile saline, i.p., 50μl;
(c) PGE_1, intracranial (anterior part of the telencephalon), 18 μg in 35 μl sterile saline;
(d) PGE_1, intracranial, 5 μg in 35 μl sterile saline;
(e) sterile saline, intracranial, 35 μl respectively.

Fish of the groups a, b, c, e were put back into the warmer chamber, and those of group d into the cooler chamber.

RESULTS

The baseline preferred temperature occupied by pumpkinseeds did not differ significantly between day and night. The preferred temperature (24-hr mean hourly occupied temperature) was 28.68 ± 1.07°C. The fish exhibited a diurnal locomotor activity rhythm with $79.7 \pm 5.6\%$ of their activity occurring during the normal 12-hr light period.

E. coli-endotoxin and saline injected intraperitoneally or intracranially produced no significant change in occupied temperature (Figs 2a, 3a, 4) or locomotor activity (Figs 2b, 3b). Those fish injected with PGE_1 were sedated and did not leave the chamber into which they were placed after the injection for about 3–3.5 hr (Fig. 3b). Sedation of an animal for a longer period in a shuttlebox of the type described above is followed by dramatic changes in water temperatures. So during the time of reduced motor activity temperature increased significantly ($P < 0.01$) 6°C above for fish which were set back into the warmer chamber or decreased significantly ($P < 0.01$) 5°C below for fish which were set back into the cooler chamber, in comparison to previously preferred temperatures (Fig. 3a).

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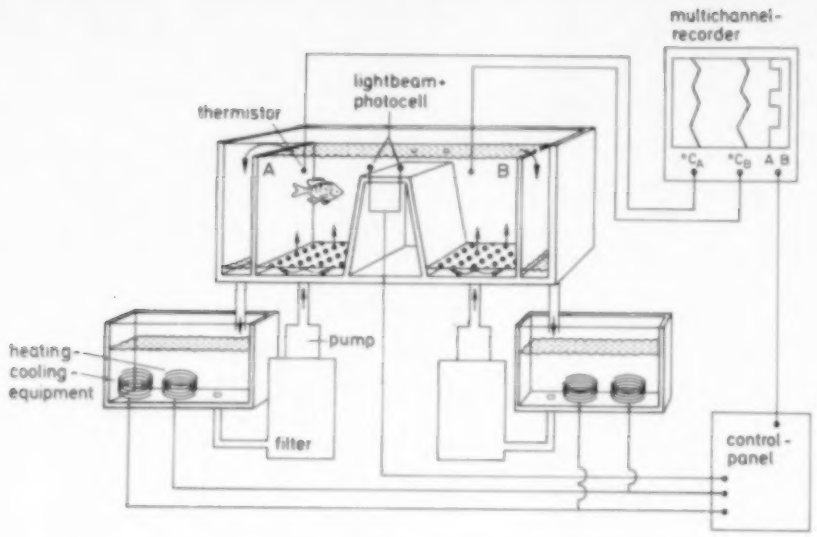

Fig. 1. Scheme of the shuttlebox. $^{\circ}C_A$, temperature of chamber A; $^{\circ}C_B$, temperature of chamber B; A and B, registration of the fish's movements between the chambers A and B (= swimming activity).

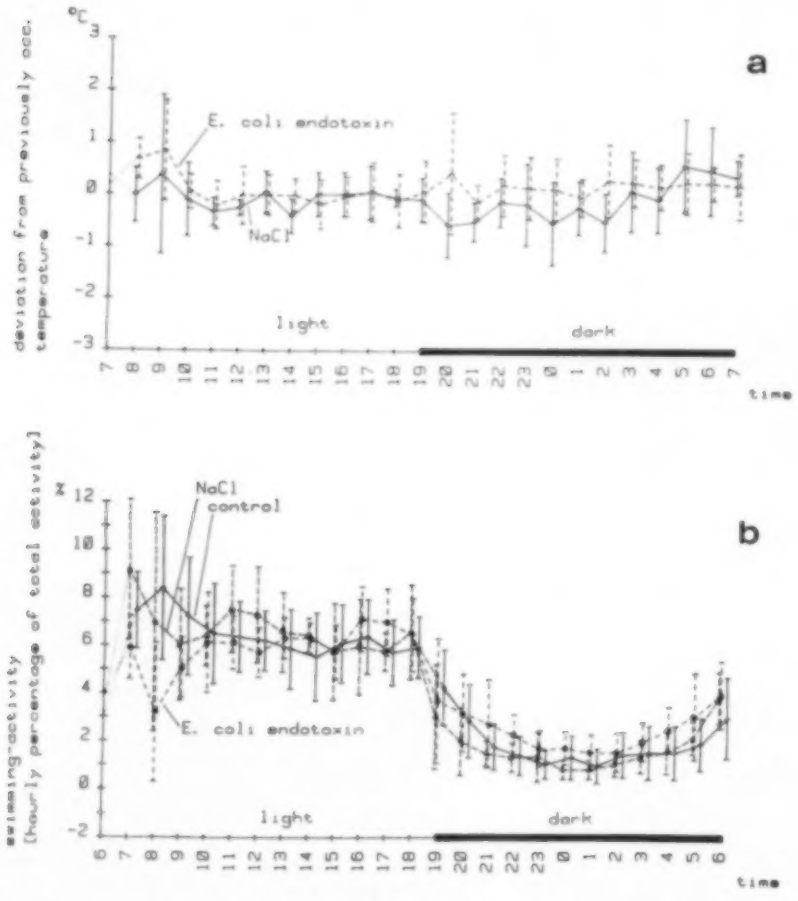

Fig. 2.(a) Differences in occupied temperatures of *Lepomis gibbosus* between control period and day of injection of saline i.p. ($N = 5$) or *E. coli*-endotoxin ($N = 8$). (b) Relative swimming-activity of *Lepomis gibbosus* at day of injection of *E. coli*-endotoxin ($N = 8$) or saline i.p. ($N = 5$).

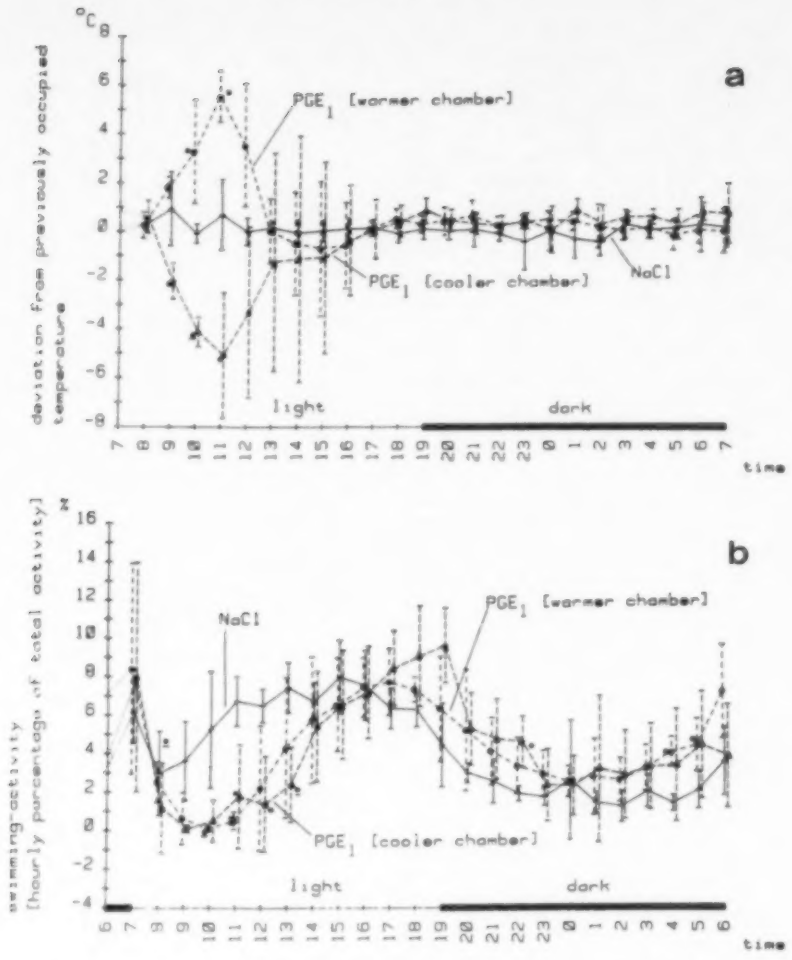

Fig. 3(a). Differences in occupied temperature of *Lepomis gibbosus* between control period and day of injection with saline i.c. ($N = 5$), and between control period and day of injection with PGE_1 (5 fish were set back into the warmer chamber and 5 fish were set back into the cooler chamber). *Differences significant ($P < 0.01$). *Differences highly significant ($P < 0.001$). (b) Relative swimming-activity of *Lepomis gibbosus* during day of injection with saline i.c. ($N = 5$) or PGE_1 (5 fish were set back into the warmer chamber and 5 fish into the cooler chamber). *Differences significant ($P < 0.01$). *Differences highly significant ($P < 0.001$).

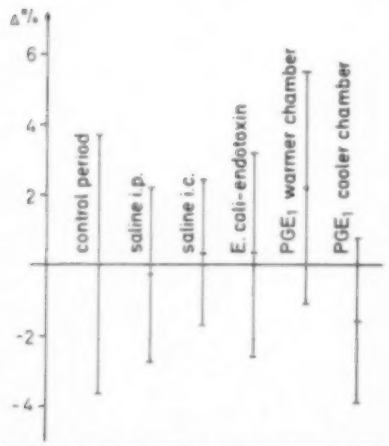

Fig. 4. Changes in preferred temperature (mean ± SD) for 24 hr of 28 *Lepomis gibbosus* relative to baseline final thermal preference.

DISCUSSION

The preferred temperatures of the 28 pumpkinseeds during control period were comparable to those found in previous studies (Richards *et al.*, 1977; Müller and Fry, 1976). Similar to the other centrarchids *Enneacanthus gloriosus*, *Lepomis macrochirus* and *Lepomis cyanellus* (Beitinger, 1974; Crawshaw, 1975; Reynolds and Casterlin, 1976; Casterlin and Reynolds, 1979b; Beitinger *et al.*, 1979), the pumpkinseeds exhibited no diurnal rhythm in preferred temperature. The higher swimming activity during daytime compared with the low activity during the night is in accordance with the results of Emery (1973; during the night *Lepomis gibbosus* was found resting at the bottom. The results for endotoxin are contradictory to those of Reynolds and his group, who found higher preferred temperatures in *Lepomis macrochirus* (2.6°C fever) and *Carassius auratus* (5.9°C fever) after injection with *E. coli*-endotoxin (Reynolds and

Casterlin, 1982). Subsequently Reynolds and co-workers found higher preferred temperatures in *Lepomis macrochirus*, *Micropterus salmoides*, *Carassius auratus* after application of *Aeromonas hydrophila* bacteria (Reynolds and Casterlin, 1982). Fletcher (1981), however, had not had "the opportunity of observing febrile reactions" in his endotoxin-injected plaice. Kuiper also could not observe fever in goldfish after injection of *E. coli*-endotoxin (0.003–20 mg/kg body wt). Probably the parameters used in his study were not discriminative enough to detect changes in preferred temperature (Kuiper, pers. comm.). Our results give evidence for the assumption that species-specific differences in the susceptibility to endotoxin similar to those in endothermic vertebrates may exist in ectotherms (van Miert and Frens, 1968; Petersen *et al.*, 1982) or that fever is not an attribute common to all vertebrates.

Fever-like phenomena following injection of PGE_1 were described in the following ectothermic species: *Rana esculenta* (Myhre *et al.*, 1977), *Necturus maculosus* (Hutchison and Erskine, 1981), two scorpion species (Cabanac and Le Guelte, 1980), *Homarus americanus*, *Penaeus duorarum*, *Limulus polyphemus* (Casterlin and Reynolds, 1979a) and *Cambarus bartoni* (Casterlin and Reynolds, 1978). In contrast to these results, according to our investigations, *Lepomis gibbous* did not show a febrile response following intracranial injection of PGE_1 (Figs 3a, 4). The observed sedative effects of PGE_1 in fish are already known from several mammals (Horton, 1964; Horton and Main, 1965; Holmes and Horton, 1968; Milton and Wendlandt, 1970; Gilmore and Saikh, 1972; Potts *et al.*, 1973; Desiraju, 1973; Haubrich *et al.*, 1973) and chicken (Asakawa and Yoshida, 1971; Nistico and Marley, 1973, 1976). Reynolds and Casterlin (1982), using a shuttlebox similar to the type used in our experiments, observed "immobilisation" in arthropods following the injection of larger doses of PGE_1. Possibly terrestrial scorpions (Cabanac and Le Guelte, 1980) and frogs (Myhre *et al.*, 1977) were sedated too and heat was transferred passively to their bodies by radiation. Hutchison and Erskine (1981) stated that *Necturus maculosus* injected with PGE_1 remained longer in the warmth than controls. So sedation as the real cause of "PGE_1-fever" in ectotherms cannot be excluded in any of the results so far reported in the literature. Kluger (1978, 1979a, b, c) and Kluger and co-workers (1975, 1977) suggested that fever has an ancient evolutionary origin and survival value. The results of Laburn *et al.* (1981) in reptiles and our results cast doubt on this theory. Nevertheless, more comparative investigations have to be done in order to clarify the possible phylogenetic origin of fever and its value for survival.

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THE INFLUENCE OF THE RATIO OF PROTEIN ENERGY TO TOTAL ENERGY IN THE FEED ON THE ACTIVITY OF PROTEIN SYNTHESIS *IN VITRO*, THE LEVEL OF RIBOSOMAL RNA AND THE RNA–DNA RATIO IN WHITE TRUNK MUSCLE OF ATLANTIC COD (*GADUS MORHUA*)

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Abstract—1. Cod (*Gadus morhua*) were fed diets containing protein energy to total energy levels (PE/TE) of 10.0, 20.6, 29.6, 38.4, 56.2 and 74.1% for 21 days.

2. Ribosomes were isolated from the white trunk muscle tissue, the capacity for protein synthesis *in vitro* determined and related to muscle tissue wet weight, rRNA and DNA.

3. Protein concentrations of less than 47.4% PE/TE in the diets reduce the ribosomal capacity for protein synthesis per g wet weight and per mg DNA, and the tissue contents of rRNA and ratio of rRNA/DNA.

4. The capacity for muscle protein synthesis *in vitro* is a significant and sensitive parameter of protein inadequacy in fish diets.

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INTRODUCTION

Protein synthesis activity in skeletal muscle tissue is sensitive to alterations in dietary composition and food intake. The content of ribosomal RNA (rRNA) in rat skeletal muscle as well as the ability of rat skeletal muscle to incorporate amino acids into protein *in vitro* is affected by protein starvation, amino acid depletion and the biological value of dietary nitrogen sources (von der Decken and Omstedt, 1970, 1972; Omstedt and von der Decken, 1972, 1974; Omstedt *et al.*, 1973). Recent studies with atlantic cod (*Gadus morhua*) (Lied *et al.*, 1982) and rainbow trout (*Salmo gairdneri*), saithe (*Pollachius virens*) and herring (*Clupea harengus*) (Rosenlund *et al.*, 1983) have demonstrated that ribosomes isolated from the white trunk muscle tissues of these fish species actively incorporate amino acids into protein, *in vitro*.

The present investigation is concerned with the effect of diets containing different ratios of protein energy to total energy on the level of protein synthesis *in vitro*, rRNA levels and the ratio rRNA/DNA in white muscle tissue from cod. The extent to which these parameters are effective indicators of protein quantity of recently ingested feeds is also studied.

MATERIALS AND METHODS

Cod (*Gadus morhua*), averaging 100 g, hatched and reared at the Aquaculture Research Station, Austevoll, western Norway, were used in the experiment, which was carried out in 175 l. dark green coloured oval fiberglass tanks supplied with running sea water at 8°C and a salinity of 35‰. The light–dark cycle was 12–12 hr. The fish were acclimatized to the experimental conditions for 14 days. During this period they were fed once daily *ad lib.* a lightly frozen (−5°C) diet (Expt diet, Group No. 5). Care was taken to use only healthy fish in the experiment.

The experimental diets were based on 80% fillet from saithe (*Pollachius virens*) containing 0.5% fat, 16.8% protein (N × 6.25) and 20% dry matter, and 20% raw krill (*Meganyctiphanes norvegica*) containing 1.4% fat, 13.2% protein and 19.1% dry matter as sources of protein (Table 1). Fish oil from capelin (*Mallotus villosus*) and dextrinized wheat starch were used as energy sources. The energy contents of the diets were calculated using metabolizable energy values of 18.0, 33.5 and 12.5 kJ/g for the metabolizable energy of protein, fat and carbohydrates, respectively (Brett and Groves, 1979). The energy level from carbohydrates was kept constant at 19.5% in all diets. The energy content from protein was balanced by fish oil addition.

Water was not added to the diets due to the water content of the protein sources. The diets were processed into moist pellets, 5 mm in diameter and a length of 4–6 mm, and stored at −25°C. The feed was offered to the fish as lightly frozen (−5°C) moist pellets.

The experiment was conducted with 7 groups, each containing 10 fish. The ratios (%) of protein energy to total energy (PE/TE) in the 7 diets are listed in Table 1. The fish were fed *ad lib.* once daily during the experimental period, which lasted for 21 days.

Collection of muscle samples

Seven or 8 fish were collected randomly from each group, killed by a blow on the head and bled. The epaxial muscle and part of the hypaxial muscle from both sides were dissected skinned and split lengthwise into several thin slices. The muscle tissue, wrapped in aluminium foil, was immediately placed between two blocks of solid CO_2 and stored at −80°C for not more than 14 days (Lund and von der Decken, 1980).

Preparation of ribosomes and liver cell sap

Ribosomes and liver cell sap was prepared as described previously (Lied *et al.*, 1982) with the modification that a pH of 7.6 was used instead of 7.8. Muscle samples from one fish of each group were analysed on the same day. Ribosomes, prepared from tissue homogenates corresponding to 0.75 g of tissue, were suspended in 1 ml of the incubation medium,

Table 1. Composition of the experimental diets

Ingredients (g/kg wet wt)	Group No. 1	2	3	4	5	6	7
Saithe fillet	305	494	577	629	665	695	730
Raw krill	76	123	144	157	167	173	182
Capelin oil	304	175	115	78	52	32	5
Dextrin from wheat starch	261	169	132	108	91	79	63
Vitamin mixture*	34	23	17	14	12	11	8
Mineral mixture†	7	6	5	4	3	2	2
Guar gum	10	10	10	10	10	10	10
kJ/g wet wt	14.92	10.03	7.77	6.40	5.39	4.68	3.68
PE/TE (%)	10.0	20.6	29.6	38.4	47.4	56.2	74.1

*Composition of the vitamin mixture (mg vitamin/kg vitamin mixture): thiamin–HCl 167; riboflavin 333; pyridoxine 167; Ca-pantothenate 667; niacin 25; folic acid 83; ascorbic acid 667; vitamin A 16400 IU; vitamin D 2680 IU; and α-tocopherol acetate 6000 IU. Dextrin was used as the main constituent of the mixture.

†Commercial standard mineral mixture used for poultry and swine containing (g/kg): phosphorus 60; calcium 240; sodium 60; magnesium 10; iron 2; manganese 2; zinc 2.5; copper 0.4; iodine 0.075; and selenium 0.008.

giving a suspension of 1.25 ml. Liver cell sap was prepared from rainbow trout (*Salmo gairdneri*) kept in tanks supplied with running sea water of 8°C and a salinity of 35‰.

Amino acid incorporation by ribosomes

The incubation mixture contained phosphocreatine 25 mM, creatine phosphokinase (EC 2.7.3.2) 0.80 units, ATP1 mM, GTP 0.1 mM, a mixture of amino acids excluding the radioactive one 0.1 mM, L-(1-^{14}C) phenylalanine (0.05 μCi) 0.006 mM, sucrose 250 mM, Tris–HCl (pH 7.6 at 25°C) 35 mM, KCl 80 mM, $MgSO_4$ 5 mM, liver cell sap corresponding to 0.5 mg protein, and 50 μl of the ribosome suspension corresponding to 15–25 μg of RNA (Omstedt and von der Decken, 1972). The final incubation volume was 130 μl. The mixture was incubated for 4 min at 28°C (Lied *et al.*, 1982). After incubation, 100 μl was transferred to filter-paper discs which were kept for 10 min in ice-cold 10% trichloroacetic acid, extracted twice for 10 min at 90°C in 5% trichloroacetic acid, thereafter washed three times in ethanol, dried and counted for radioactivity in a scintillation spectrometer.

Analyses

RNA was extracted from the ribosomes in 0.4 M $HClO_4$ for 18 min at 70°C, and the absorbance measured at 260 nm. RNA was calculated on the basis of 34.2 optical density units at 260 nm/mg of RNA. DNA was analysed by a fluorometric method (Setaro and Morley, 1976) using salmon DNA as a standard.

Statistical evaluation

Four determinations were made with each ribosome suspension to assess the amino acid incorporating activity. Regression lines were fitted by the least square method (Snedecor, 1956). The capacity for protein synthesis was expressed as activity per mg of ribosomal RNA, per g wet weight of muscle, and per mg of DNA. The results are presented as the mean values ± standard error of the mean (SEM).

RESULTS AND DISCUSSION

The required optimum levels of protein in fish diets have been estimated for several species of juvenile fish. Such studies, mainly based on the minimum dietary protein level producing the maximum growth or protein retention in fish, have shown that fish are more demanding in their requirement of protein and thus for essential amino acids than birds and mammals. Estimated dietary protein requirements of different fish species, including rainbow trout (*Salmo gairdneri*), chinook salmon (*Oncorhynchus tschawytscha*), carp (*Cyprinus carpio*), eel (*Anguilla japonica*) and plaice (*Pleuronectes platessa*) have been listed by Cowey (1975) and show that crude protein levels in the feed for optimal growth varies between 380 and 500 g/kg. It has been shown that the protein content of the diet affects the capacity for protein synthesis by rat skeletal muscle ribosomes (von der Decken and Omstedt, 1970, 1972; Omstedt and von der Decken, 1972). Growth is accomplished primarily by protein synthesis. Since muscle tissue accounts for approximately 50% of total fish weight, measuring the protein synthesis capacity in muscle might be used as a measure of growth rate of the fish.

Protein concentrations of less than 47.4% PE/TE in the cod diets reduced the ribosomal capacity for protein synthesis per g wet weight per min as well as per mg DNA per min (Table 2). As the dietary concentration of protein increased from 10.0 to 47.4% PE/TE, the incorporating activity *in vitro* was more than doubled from 27.35 ± 2.65 to 62.87 ± 4.24 pmol ^{14}C-Phe incorporated into protein per min per g wet weight, and from 34.66 ± 3.75 to 83.49 ± 5.01 pmol ^{14}C-Phe incorporated into protein per min per mg DNA. A further increase of protein in the diets did not affect the incorporating capacity, which remained approximately constant at 62 and 84 pmol ^{14}C-Phe incorporated per min per g wet weight and per min per mg DNA, respectively. The effect of increasing levels of PE/TE (%) was less pronounced when expressed in terms of specific activity of rRNA (pmol ^{14}C-Phe incorporated into protein/min/mg rRNA). The specific activity increased with increasing levels of protein in the diet up to 29.6% PE/TE after which it remained relatively constant with values between 78 and 87 pmol ^{14}C-Phe inc./min/mg rRNA.

The contents of DNA in muscle tissues did not differ significantly between any of the experimental groups, but varied between 0.73 and 0.79 mg DNA/g wet weight (Table 3). As the DNA content in diploid cells is constant within species and relates to number and size of chromosomes, DNA concentrations generally reflect cell size and number of cells per unit wet weight of tissue. Thus the feeding of different levels of PE/TE (%) to cod did not significantly ($P < 0.05$) alter the cell volume or cell number per g wet weight

Table 2. Effect of the ratio of PE/TE in feed on the *in vitro* amino acid incorporating activity of ribosomes isolated from white trunk muscle of cod.

Group No.	PE/TE (%)	Number of fish	pmol ^{14}C-Phe incorporated into protein per min per		
			g wet wt	mg rRNA	mg DNA
1	10.0	8	27.35 ± 2.65	56.39 ± 5.83	34.66 ± 3.75
2	20.6	8	42.46 ± 4.26	74.49 ± 7.64	54.92 ± 4.93
3	29.6	7	48.94 ± 4.06	83.23 ± 6.16	66.76 ± 5.51
4	38.4	8	59.24 ± 4.17	80.80 ± 2.62	74.80 ± 7.20
5	47.4	8	62.87 ± 4.24	78.19 ± 5.20	83.49 ± 5.01
6	56.2	8	61.87 ± 5.76	87.14 ± 4.52	82.38 ± 9.04
7	74.1	8	62.45 ± 4.04	83.26 ± 4.23	83.26 ± 6.66

All values are mean ± SEM.

in the white muscle tissue within the present experimental period used. However, the slightly higher average values of DNA, which were found in muscle tissues of the fish fed low, as compared with the fish fed high, levels of protein may indicate differences in cell diameter due to a reduced synthesis of myofibrillar proteins. Rosenlund *et al.* (1983) have shown that proteins of myofibrillar origin were predominant in the ribosome fraction. The amount of RNA per g wet weight of muscle increased from 0.485 ± 0.017 mg in fish fed 10.0% PE/TE to 0.804 ± 0.026 mg in fish fed 47.7% PE/TE in the diet and accompanied the increase of ^{14}C-Phe incorporated into protein per g wet weight and per mg DNA (Table 3). The ribosomal RNA accounts for 80–90% of total RNA in skeletal muscle tissue of rat (Young, 1970). Von der Decken and Omstedt (1972) found that the RNA present in the ribosomal preparation of rat skeletal muscle prepared by the same methods as used in our study was of ribosomal type. Assuming that these relationships exist in fish muscle tissue, our results show that the amount of ribosomes per muscle cell was reduced in fish fed diets containing levels with PE/TE less than 47.4, as indicated by the ratios of RNA to DNA (rRNA/DNA) (Table 3). A strong correlation is thought to exist in most vertebrate tissues between RNA content and rate protein synthesis, (Munro 1969, 1976; Millward *et al.*, 1976). The values of pmol ^{14}C-Phe incorporated into proteins per min per g wet weight of muscle tissue of the 55 individual fishes used in the present experiment were related to their corresponding values of rRNA content (Fig. 1) and ratios of rRNA/DNA (Fig. 2). The *in vitro* protein synthesizing activity in skeletal muscle of fish is highly correlated to its content of rRNA ($r = 0.78$, $P < 0.001$) and its ratio of rRNA/DNA ($r = 0.70$, $P < 0.001$). Thus the protein synthesis capacity of muscle tissue to a major extent depends on the amount of rRNA in the cell and consequently on the rate of RNA metabolism (both synthesis and breakdown), changing in response to the amino acid supply (see Munro, 1976; von der Decken, 1983).

Although protein concentrations in the feeds less than 47.4% PE/TE affected the incorporating capacity per g wet weight and per mg DNA, the specific activity per mg RNA was found to be reduced only in muscle tissue of fish fed 10.0 and 20.6% PE/TE (Table 2). The reduced incorporating activities in muscle tissues of fish fed the 29.6 and 38.4% PE/TE diets as compared with the fish fed the 47.4 to 74.1% PE/TE diets may be due only to the reduced levels of rRNA, while additional effects of an overall amino acid deficiency such as polyribosomal disaggregation (Munro, 1976; Clemens and Pain, 1976) reduce the rate of protein synthesis further in muscle tissues of the fish fed 10.0 and 20.6% PE/TE. Von der Decken (1977) found that peptidyltransferase activity should be considered as one of the factors influencing the capacity of polyribosomes to synthesize proteins during a restricted protein supply. Thus, in cod fed low levels of protein the activity of ribosomes per unit wet weight of tissue is a function of both their specific activity and content.

Taken together, when the feed protein is of a high quality regarding digestibility and amino acid composition, 45–50% of the energy in cod feed should be supplied by protein to obtain a maximal protein synthesizing activity in muscle tissue of cod. The proteins used in our experimental diets were composed of 80% of saithe fillet and 20% of krill (Table 1) and should be considered of high quality with respect to amino acid composition relative to the requirement of cod. The apparent availability of protein ($N \times 6.25$) and amino acids from saithe fillet have been estimated to 91.6 and 96.2%, respectively (Lied and Njaa, 1982). Fish, like other animals, eat to satisfy their energy requirement (Lee and Putnam, 1973). The required protein level in the feed should

Table 3. Effect of the ratio of PE/TE in feed on the content of ribosomal RNA and DNA in white trunk muscle of cod

Group No.	PE/TE (%)	Number of fish	rRNA (mg/g wet wt)	DNA (mg/g wet wt)	rRNA/DNA
1	10.0	8	0.485 ± 0.017	0.789 ± 0.043	0.614 ± 0.026
2	20.6	8	0.570 ± 0.035	0.773 ± 0.040	0.737 ± 0.037
3	29.6	7	0.588 ± 0.030	0.733 ± 0.017	0.802 ± 0.048
4	38.4	8	0.734 ± 0.046	0.792 ± 0.048	0.926 ± 0.074
5	47.4	8	0.804 ± 0.026	0.753 ± 0.034	1.067 ± 0.063
6	56.2	8	0.710 ± 0.060	0.751 ± 0.026	0.946 ± 0.091
7	74.1	8	0.750 ± 0.051	0.750 ± 0.022	1.000 ± 0.088

All values are mean ± SEM.

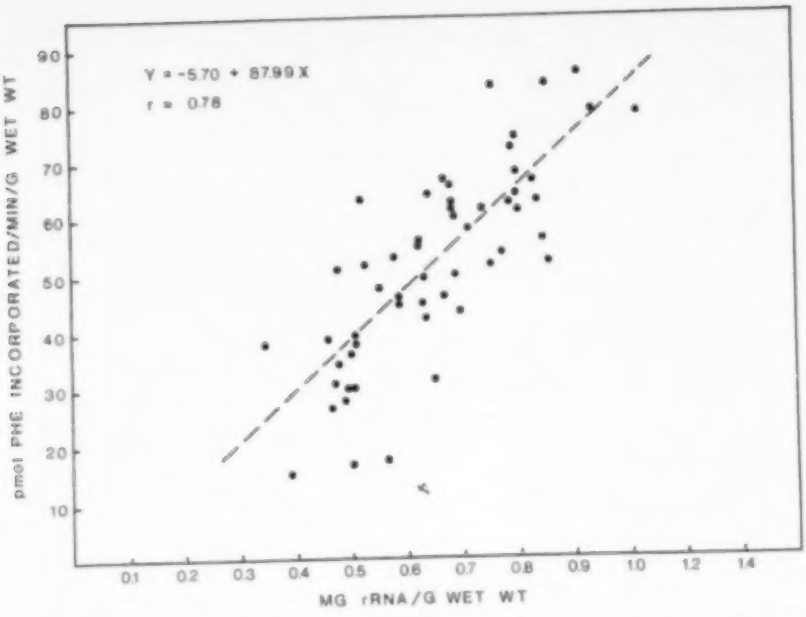

Fig. 1. Linear correlation between pmol ^{14}C-Phe incorporated into proteins per min per g wet weight and mg rRNA per g wet weight of white muscle tissue of cod.

therefore be related to its total content of energy. However, satiety may also be a matter of stomach filling when fish are fed diets of high caloric density and thus of low volume per energy unit. This may explain the increasing intake of energy with the decreasing levels of protein in feed, especially at low PE/TE (%), found in the present experiment (Table 4).

Regression analyses by the least square method was performed from the actual percentage levels of PE/TE (%) in the diets of the experimental groups fed the 10.0, 20.6, 29.6, 38.4 and 47.4% PE/TE diets and the corresponding mean values of pmol ^{14}C-Phe incorporated into proteins per g wet weight of mg rRNA and the ratios rRNA/DNA (Table 5). Several different curve fits were tested. The percentage levels

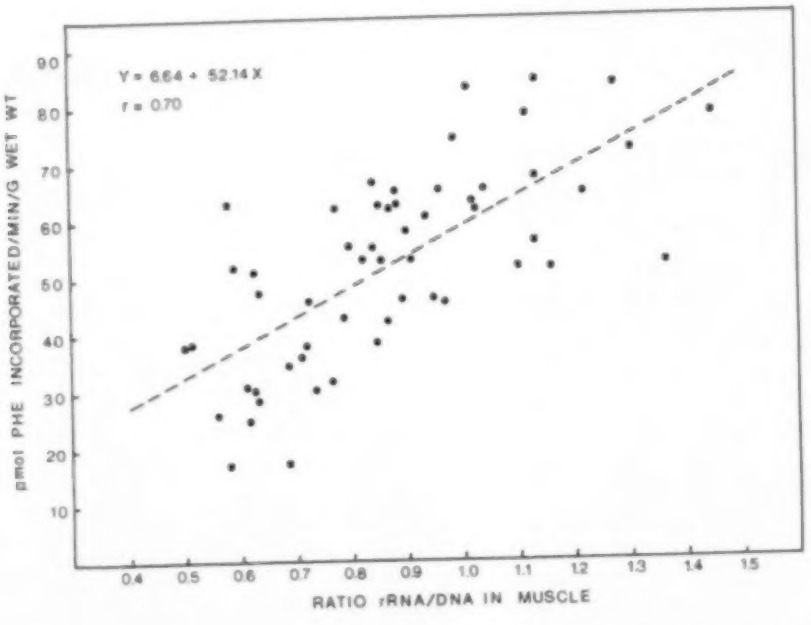

Fig. 2. Linear correlation between pmol ^{14}C-Phe incorporated into proteins per min per g wet weight and the ratio of rRNA to DNA in white muscle tissue of cod.

Table 4. Total intake of energy (MJ) and protein (N × 6.25) of the groups fed different levels of PE/TE during an experimental period of 21 days

Group No.	Energy intake (MJ)	Protein intake (g)
1	8.43	47
2	7.29	84
3	5.25	87
4	4.86	103
5	4.82	127
6	4.80	149
7	4.20	171

of PE/TE (Y) and pmol amino acid incorporated per min per g wet weight (X) were best fitted ($r = 0.99$) by a power function, whereas the percentage levels of PE/TE (Y) and the contents of rRNA (X), as well as the ratios of rRNA/DNA (X), were best fitted by hyperbolic functions ($r = 0.98$ and 0.99, respectively) (Table 5). Given an adequate sample size with a reliable population mean, all three variables provided a measure of the quantity of protein in the feed. Several studies have been performed using the concentration of RNA and the ratio of RNA to DNA as indicators of recent change in body or tissue size due to growth or various feeding conditions (Sutcliffe, 1969; Bulow, 1970; Haines, 1973; Cowey *et al.*, 1974; Emmersen and Emmersen, 1976; Kayes, 1978; Buckley, 1979; Bulow *et al.*, 1981). Measurement of the *in vitro* protein synthesis activity has been introduced as a method of nutritional evaluation of protein (von der Decken and Omstedt, 1972; Omstedt *et al.*, 1973; von der Decken *et al.*, 1975). Recently the authors found that the *in vitro* ribosomal capacity for protein synthesis in white trunk muscle of cod starved for 10 days was considerably reduced as compared to fed cod (Lied *et al.*, 1982). The data of the present experiment shows that the capacity for muscle tissue protein synthesis *in vitro* is a significant and sensitive parameter of protein inadequacy in fish diets.

The ability of a feed protein to support protein synthesis is related to its digestibility and its amino acid composition relative to the animal requirement. Data on *in vitro* protein synthesis in skeletal muscle of rat correlates closely with the digestibility of feed proteins (von der Decken *et al.*, 1975). As compared with the variables mg RNA per g muscle tissue and rRNA/DNA, changes in both ribosomal specific activity and ribosomal content are considered when results are expressed as pmol amino acid incorporated into protein per g wet weight of muscle. Thus, the proposed technique has the advantage of measuring the total effectiveness of a feed protein to supply amino acids for growth-related intracellular functions.

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Table 5. Regression line equations fitted by the least square method from the actual percentage levels of PE/TE (‰) in the diets of the experimental groups fed 10.0, 20.6, 29.6, 38.4 and 47.4‰ PE/TE diets, and the corresponding mean values of pmol amino acid incorporated into proteins per g wet weight, of mg rRNA, and of the ratios rRNA/DNA

Y	X	Regression line equations	r
PE/TE (‰)	pmol ^{14}C-Phe/g wet wt	$Y = 0.023(X^{1.83})$	0.99
	mg rRNA/g wet wt	$Y = 100.7 + (-44.0/X)$	0.98
	rRNA/DNA	$Y = 97.8 + (-54.9/X)$	0.99

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EFFECTS OF OXYGEN DEPRIVATION DURING ANOXIA AND MUSCULAR WORK ON THE ENERGY METABOLISM OF THE CRAYFISH, *ORCONECTES LIMOSUS*

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Abstract—1. During environmental anoxia glycogen was the sole substrate and L-lactate the only quantitatively important anaerobic end product in the crayfish *Orconectes limosus*.

2. Muscle lactate concentration increased to 20 mM, whereas blood lactate level accumulated to approx. 60 mM after 16 hr of anoxia.

3. A pronounced breakdown of arginine phosphate and a decrease of the energy charge value occurred only during prolonged anoxia.

4. During recovery from anoxia venous P_{O_2} was elevated, the phosphagen pool restored between 1 and 6 hr and blood and muscle lactate levels were not back to control levels between 12 and 24 hr.

5. During functional anoxia (swimming) muscle phosphagen level was diminished rapidly, some lactate was produced and the energy charge dropped from 0.94 to 0.79.

6. During recovery after exercise venous hemolymph P_{O_2} rose, the energy charge was fully restored and the phosphagen pool was restored to 70% (1 hr) and muscle and blood lactate levels were slightly elevated

INTRODUCTION

Reduced ambient concentrations of oxygen resulting in hypoxic or even anoxic conditions may be encountered by many freshwater species. The decapod crustacean *Orconectes limosus* has successfully inhabited biotopes such as polluted streams and ponds and the mud zone of slow flowing rivers and lakes, where oxygen partial pressure may be low. Although *O. limosus* is a species of the suborder Reptantia (animals that have become highly adapted to crawling), this crayfish still possesses a large extended abdomen and is capable of rapidly swimming backwards by flapping its tail. High-speed swimming is characteristic of the escape response which is achieved by ventral flexion of the abdomen (Webb, 1979). During excessive swimming activity, oxygen tensions in the abdominal muscle may become limiting and the animal has to cope with functional anaerobiosis. Although environmental and functional anaerobiosis have been studied in numerous bivalves and annelids (for recent reviews, see Gäde, 1980a; Schöttler, 1980; Livingstone, 1982; Gäde, 1983a), only a few investigations have been performed on crustaceans. Most authors have studied anaerobic metabolism during activity *or* hypoxia (but not both) by measurements of lactate as the main end product, either in the hemolymph (Phillipps *et al.*, 1977, in the Australian crayfish *Cherax destructor*; Bridges and Brand, 1980, various species of crustacea; Burke, 1979, in the intertidal crabs *Pachygrapsus crassipes* and *Carcinus maenas*; Onnen and Zebe, 1983, in the shrimp *Crangon crangon*) or in the tail muscle (Phillipps *et al.*, 1977; Onnen and Zebe, 1983). Other authors have carried out experiments on anaerobic metabolism during anoxia in mud-dwelling decapod shrimps, *Upogebia pugettensis* and *Callianassa californiensis*, by measuring hemolymph lactate concentrations (Pritchard and Eddy, 1979) or lactate and other anaerobic metabolites in whole animals (Zebe, 1982). Similar experiments have been described for the scavenging isopod *Cirolana borealis* by de Zwaan and Skjoldal (1979). There is little evidence in the literature of investigations of metabolites in the tail muscle and hemolymph after both environmental and functional anaerobiosis. In addition, it is not known whether, and to what extent, the phosphagen of the muscle tissue, arginine phosphate, contributes to energy production during these conditions of oxygen deprivation. Recently it has been established for bivalves and annelids that the phosphagens are important for ATP production, during burst muscular activity as well as during anoxia survival (Surholt, 1977; Ebberink *et al.*, 1979; Gäde, 1980b; Meinardus and Gäde, 1981).

Accordingly, the subject of the present study was to delineate concentration changes of substrates and metabolic end products in the tail muscle and hemolymph of *O. limosus* during and after environmental and functional anaerobiosis.

MATERIALS AND METHODS

Crayfish

Specimens of *Orconectes limosus* were obtained from the Havel River in Berlin, kept at Bonn in large tanks in well-aerated (P_{O_2}: 135 mmHg) running tap water at 13 ± 2°C. They were fed on commercially available cat food pellets once a week, but kept unfed for about 2 days before experimentation. Experimental animals were in the intermolt stage, weighed about 22 ± 6 g (wet weight), and had a total length of about 12 ± 2 cm.

Chemicals

Most enzymes, coenzymes, substrates and the adenine nucleotides were purchased from Boehringer, Mannheim, or from Sigma GmbH, München. All other chemicals were of analytical grade and obtained from Merck, Darmstadt. Octopine dehydrogenase was prepared from the adductor muscle of *Pecten maximus* as previously described (Gäde and Head, 1979).

Experimental procedure

Anoxic conditions were obtained by gassing a Plexiglas respiration chamber filled with 15 l of tap water with pure nitrogen and monitoring the P_{O_2} with an oxygen electrode (E 5046, Radiometer, Copenhagen). The electrode was calibrated with air-saturated water and with water completely depleted of oxygen by addition of Na_2SO_3 solution. Five to ten animals were placed in the chamber which was closed and further flushed with pure nitrogen for 15 min before the chamber was closed. It was then left undisturbed in a constant temperature room (14 ± 2°C) for the remainder of the experiment. At the end of the incubation period, the animals were removed, the tails cut off, the muscle tissue dissected, blotted on filter paper and immediately frozen in liquid nitrogen. The entire process took less than 45 sec.

Swimming was achieved by prodding individuals in a large tank (300 l) with well-aerated tap water (13 ± 2°C). The number of tail flips was counted and the time required was monitored. The experiment was terminated when a crayfish had ceased swimming voluntarily. Dissection of the tail muscle was as above.

Hemolymph sampling was done in the following way: after removal of the animal from the tank a 300–500 µl prebranchial blood sample was drawn into a 1 ml plastic syringe from the infrabranchial sinus through the arthrodial membrane above the base of a walking leg. The hemolymph was immediately blown into a preweighed Eppendorf vial containing 6% perchloric acid (for lactate determination) and the remaining was analysed immediately for P_{O_2} with an oxygen electrode housed in a thermostatted (14 ± 2°C) cell (D 616, Radiometer, Copenhagen).

Preparations of extracts and analysis of metabolites

Perchloric acid extracts of the frozen tissue were prepared as described previously (Gäde *et al.*, 1978) and all metabolites were assayed enzymatically according to the following methods: ATP (Lamprecht and Trautschold, 1974); ADP and AMP (Jaworek *et al.*, 1974); arginine phosphate (Grieshaber *et al.*, 1978); arginine (Gäde and Grieshaber, 1975); L-alanine (D. H. Williamson, 1974); D-alanine (Graßl, 1974); L-lactate (Boehringer: Methoden der enzymatischen Lebensmittelanalytik, 1980); succinate (J. R. Williamson, 1974); aspartate (Bergmeyer *et al.*, 1974). Glycogen was measured enzymatically with amyloglucosidase after hydrolysis in boiling KOH (30%) and subsequent alcohol precipitation as described by Keppler and Decker (1974).

All values are given as µmol/g wet weight, since the water content of the tail muscle did not change significantly during the experimental period; the dry weight of 15 randomly chosen individuals (five control animals and ten animals subjected to different periods of anoxia) was 21.6 ± 2.8% of the wet weight.

Remarks on the enzymatic assay of L-lactate in the hemolymph

Recently Graham *et al.* (1983) reported that no stable end point of the enzyme analysis (further change of NADH at 340 nm) could be obtained after 45 min using glycine–hydrazine buffer (pH 9.2) for the determination of L-lactate in the hemolymph of the crustaceans *Callinectes sapidus* and *Cancer magister*. The phenomenon was suggested—as pointed out earlier by Engel and Jones (1978)—to be due to the liberation of Cu ions into the solvent when hemocyanin is precipitated by perchlorate; it was eliminated by lowering the pH of the buffer to 9.0 with HCl and adding EDTA (12 mM). We avoided this problem by using a glycylglycin buffer (0.6 M glycylglycin, 0.1 M L-glutamate, pH 10.0) and glutamate–pyruvate-transaminase instead of a hydrazine buffer in order to eliminate pyruvate from the equilibrium. With this method, no interference with heavy metal ions was observed and a stable end point was always approached within 10–15 min.

RESULTS

Anaerobic metabolism during experimental environmental anoxia

In our first experiments (November 1980 to January 1981) we determined the concentrations of the adenine nucleotides, the phosphagen pool, the stored glycogen, and the levels of known anaerobic end products in the tail muscle of animals exposed to 16 hr anoxia (Table 1). As no changes in the levels of adenine nucleotides were observed up to 12 hr of anoxia the mean values from all groups (control to 12 hr) are presented. No obvious decrease of the energy charge value was observed during this period. Between 12 and 16 hr of anoxia the energy charge dropped from 0.94 to 0.82 which was due to the decrease in the ATP concentration and of the increase of the ADP and AMP levels, respectively. Also, the levels of arginine phosphate (decrease) and arginine (increase) changed significantly only after 12 and 16 hr of anoxia, when the arginine phosphate pool was calculated to represent about 40 and 30% of the total pool of arginine phosphate and arginine (controls: 60%). Both stereoisomers of alanine were present in the tail muscle, but, as for succinate, only a rather small change was determined during anoxia. Since virtually no change in the concentration of aspartate was found, glycogen seems to be the sole substrate during anoxia and about 50% of the glycogen was consumed during the 16 hr incubation period. L-Lactate, which was as low as 1.25 µmol/g wet weight in controls, was the major anaerobic end product and increased gradually up to 12 hr of anoxia when it levelled off between 16 and 19 µmol/g wet weight.

Changes of the prebranchial P_{O_2} and L-lactate levels in the hemolymph during environmental anoxia

The effect of various periods of anoxia on the oxygen tension in the prebranchial blood and the L-lactate concentration is shown in Fig. 1. The venous control P_{O_2} (10.5 mmHg) is in the same order of magnitude as the values for other species of crayfish at a comparable temperature: *Austropotamobius pallipes*, 17 mmHg (Wheatly and Taylor, 1981); *Pacifastus leniusculus*, 4.4 mmHg (Rutledge, 1981); *Astacus leptodactylus*, 7.2 mmHg (Angersbach and Decker, 1978). In *O. limosus*, blood P_{O_2} decrease to very low values during anoxia as was observed during hypoxia (inspired oxygen tension of 63 mmHg) in *A. pallipes* (Wheatly and Taylor, 1981).

Blood L-lactate concentrations in *O. limosus* showed a dramatic 90-fold increase during the incubation period attaining a maximal concentration of

Table 1. Concentrations of metabolites (μmol/g wet weight; glycogen: μmol glycosyl units/ g wet weight) in the tail muscle of *O. limosus* during environmental anoxia*

Hours of anoxia	ATP	ADP	AMP	E.C.	Arg-P	Arg	Arg-P in % of Arg-P + Arg	Glycogen	L-Lactate	Alanine L-	Alanine D-	Succinate
0	4.09 ± 1.63†	0.41 ± 0.17†	0.06 ± 0.02†	0.94 ± 0.01	20.8 ± 2.9	14.4 ± 3.5	59.1	77 ± 28	1.25 ± 0.59	2.14 ± 0.76	1.73 ± 0.42	0.15 ± 0.04
1				0.91 ± 0.01	17.7 ± 3.2	10.6 ± 3.3	62.5		2.46 ± 1.18	2.21 ∓ 0.91	3.24 ± 1.64	0.20 ± 0.06
4				0.92 ± 0.04	15.0 ± 4.3	13.3 ± 2.3	53.0		5.97 ± 2.14	2.38 ± 0.98	2.37 ± 0.86	0.18 ± 0.04
8				0.93 ± 0.02	15.7 ± 4.0	14.4 ± 2.6	52.2		8.77 ± 2.43	2.30 ± 0.97	3.04 ± 0.99	0.47 ± 0.14
12				0.93 ± 0.01	15.6 ± 3.6	23.7 ± 2.6	39.7		19.27 ± 5.20	2.77 ± 1.43	3.64 ± 1.54	0.56 ± 0.18
16	3.75 ± 0.82	1.12 ± 0.07	0.36 ± 0.13	0.82 ± 0.04	11.4 ± 4.9	24.4 ± 7.4	31.8	39 ± 13	16.31 ± 5.40	2.58 ± 0.43	3.98 ± 1.05	1.28 ± 0.01

*Mean value ± SD of four individual animals.
†Since no significant change in the levels of adenylates occurred after up to 12 hr of anoxia the mean of all individuals of these groups ($N = 20$) is given and only the E.C. for the different groups is given separately.

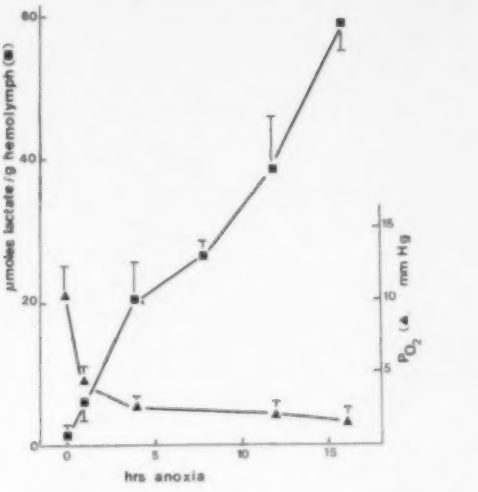

Fig. 1. The effect of experimental (environmental) anoxia on the levels of hemolymph P_{O_2} (given as mmHg ± SD) and L-lactate (μmol/g hemolymph ± SD).

about 60 mM. The accumulation curve had a biphasic time course with fast accumulation rates during the first 4 hr and between 12 and 16 hr of anoxia.; the average accumulation rate was calculated to be 3.62 μmol L-lactate per hr and g hemolymph.

Recovery from environmental anoxia

In the second experiment (March/April 1982) we analysed the concentrations of arginine phosphate, arginine, L-lactate, D- and L-alanine and succinate in the tail muscle during recovery from 12 hr anoxia in aerobic water (Table 2). Since no changes in the levels of adenylates occurred during anoxic conditions of this length, these parameters were not measured. The controls for this experiment (12 hr anoxia) showed very similar changes as previously illustrated in Table 1. Restoration of the phosphagen pool took more than 1 hr, but was accomplished after 6 hr of recovery (about 60% of the total arginine phosphate plus arginine pool) with a concomitant decrease of the arginine concentrations. The L-lactate level, on the other hand, was still high after 6 hr, and even after 12 hr of recovery 5.6 μmol/g wet weight were present. The levels of the alanines and succinate were also back to control values after 1 and 6 hr, respectively.

Changes of prebranchial P_{O_2} and L-lactate levels in the hemolymph during recovery from environmental anoxia

Figure 2 depicts the effect of recovery on the levels of blood P_{O_2} and L-lactate in the hemolymph. Up to 3 hr of recovery the venous P_{O_2} was increased to 40–50 mmHg, dropped about 50% in the next hours, and was back to the control level after 24 hr of recovery (Fig. 2 vs Fig. 1). The blood L-lactate level remained high up to 3 hr of recovery and dropped subsequently. The rate of L-lactate disappearance from the hemolymph was faster between 3 and 6 hr of recovery.

Table 2. Concentrations of metabolites (μmol/g wet weight) in the tail muscle of *O. limosus* during recovery after 12 hr of anoxia

Hours of recovery	Arg-P	Arg	Arg-P in % of Arg-P + Arg	L-Lactate	Alanine L-	Alanine D-	Succinate
0 = 12 hr anoxia	16.6 ± 3.2	33.2 ± 8.7	33.3	18.7 ± 4.3	5.3 ± 2.3	3.9 ± 1.8	1.1 ± 0.4
1	18.5 ± 5.1	27.1 ± 3.2	40.6	14.5 ± 4.4	3.7 ± 1.2	2.8 ± 1.4	1.0 ± 0.3
6	25.9 ± 3.0	17.9 ± 3.6	59.1	7.7 ± 0.7	4.0 ± 1.2	3.7 ± 1.8	0.7 ± 0.4
12	—	—	—	5.6 ± 0.8	—	—	—

*Mean values ± SD of four individual animals.

Anaerobic metabolism during functional anaerobiosis

The experiments were conducted mainly between March and July 1982. Since no major changes in the adenylates and phosphagen levels occurred in control animals, values of Table 1 are used for this set of data.

When the crayfish swam with a work rate of 2 tail flips per sec for 20 sec (Table 3, line 2) the energy charge and hemolymph P_{O_2} and L-lactate levels were not significantly changed; however, the muscle phosphagen content was diminished by about 60% and muscle lactate levels were increased. Recovery for 37 min (line 3) restored the phosphagen pool and muscle lactate concentrations to some extent and hemolymph lactate levels were somewhat higher as was the venous P_{O_2}.

Crayfishes that were not as intensively exercised during the same time period (frequency of tail flips was 1.1 for about 25 sec; line 4) showed only a breakdown of the phosphagen (about 50%), but no significant muscle lactate accumulation. Recovery for 15 min (line 5) was not long enough to reverse any of the changes, besides a slightly higher level of muscle lactate was determined and venous P_{O_2} was high.

Exercise with the same low frequency but for a longer time period (1.1 tail flips per sec for 1 min; line 6) resulted in a fall in the energy charge, a decrease of the phosphagen to 24% of the control value, and a slight elevation of muscle lactate levels, but no change in the hemolymph lactate was detected. Recovery for 1 hr (line 7) restored the energy charge completely and the phosphagen pool to about 70% of the control. However, both muscle and blood lactate levels and venous P_{O_2} were increased.

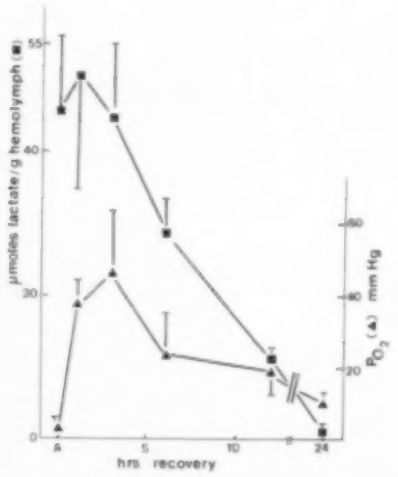

Fig. 2. Changes in the levels of hemolymph P_{O_2} (given as mmHg ± SD) and L-lactate (μmol/g hemolymph ± SD) during recovery after a period of 12 hr of environmental anoxia.

DISCUSSION

It has frequently been demonstrated that the extent of anaerobiosis depends on the degree of oxygenation of the environment (for early reviews see von Brand, 1944, 1945). For crayfishes, Park (1945) reported that pond-living species of the genus *Cambarus* are more resistant to low oxygen tensions than stream-living individuals, and as early as 1941 it was reported by Vallin (cited in Lindroth, 1950) that crayfishes can survive for 20 hr under experimental conditions when the oxygen concentrations of the surrounding medium was 0.2 mg/l. In our experiments *O. limosus* were able to tolerate zero oxygen tensions at 14°C for at least 16 hr. In a related species, *O. virilis*, exposure to ambient oxygen tensions below 30 mmHg at 18–21°C was followed by a rapid decrease in both circulatory and respiratory pumping rates and "death resulted from prolonged exposure" (McMahon *et al.*, 1974). In the British crayfish *A. pallipes*, the critical oxygen tension for maintenance of control oxygen uptake was 40 mmHg at 15°C (Wheatly and Taylor, 1981). When animals were submerged in hypoxic water with an ambient P_{O_2} of 63 mmHg, anaerobic metabolism was not involved in energy production as judged by the absence of any increase in hemolymph lactate concentration. However, lowering the ambient P_{O_2} to 36 mmHg resulted in a slight increase of hemolymph lactate level from 0.40 (normoxic) to 0.97 mM. On the other hand, if unrestrained, *A. pallipes* will migrate from hypoxic water into air when ambient oxygen tension is lowered to about 40 mmHg at 15°C (Taylor and Wheatly, 1980). Initially, the ability of the tissues to transport oxygen is impaired and hemolymph lactate concentration is elevated strongly from 0.55 mM in submerged animals to 8.28 mM after 2 hr of air exposure. However, after 24 hr of air exposure, the level of hemolymph lactate was reduced to 0.57 mM, suggesting that a high O_2 uptake was achieved by utilisation of aerial oxygen (Taylor and Wheatly, 1981).

In our study it is concluded that *O. limosus* was not able to take up oxygen from the beginning of the experiment. The available oxygen reserve in the hemolymph was rapidly consumed as can be deduced from the decline in the venous P_{O_2} after the first hour of anoxia. On this basis, the animals have to rely on

Table 3. Concentrations of metabolites (μmol/g wet weight) in the tail muscle of *O. limosus* and hemolymph P_{O_2} (mmHg) and L-lactate levels during swimming and a subsequent recovery period

Number of tail flips	Time of swimming (sec)	Tail flips per sec	Time of recovery (min)	ATP	ADP	AMP	E.C.	Arg-P	Arg	Arg-P in % of Arg-P + Arg	Lactate	Hemolymph Lactate	Hemolymph P_{O_2}
0	0	0	0	4.09 ± 1.65	0.41 ± 0.17	0.06 ± 0.02	0.94	20.8 ± 2.9	14.4 ± 3.5	59.1	1.25 ± 0.59	0.65 ± 0.36	10.5 ± 2.1
39 ± 9	20 ± 7	2.0	0	6.83 ± 1.20	0.98 ± 0.12	0.09 ± 0.02	0.93	10.8 ± 1.6	33.8 ± 2.6	24.2	6.95 ± 1.98	0.30 ± 0.11	10.8 ± 1.9
33 ± 4	18 ± 3	1.8	37	—	—	—	—	15.4 ± 2.9	26.4 ± 2.1	36.8	2.72 ± 1.14	1.42 ± 0.57	33.5 ± 4.5
27 ± 6	25 ± 5	1.1	0	4.45 ± 0.53	0.80 ± 0.15	0.13 ± 0.06	0.90	10.8 ± 2.6	26.5 ± 3.0	29.0	2.27 ± 2.03	0.62 ± 0.33	8.5 ± 1.9
35 ± 8	28 ± 13	1.3	15	4.97 ± 1.67	0.82 ± 0.08	0.14 ± 0.07	0.91	9.8 ± 6.5	30.1 ± 1.3	24.5	3.79 ± 2.00	1.24 ± 0.84	51.1 ± 19.5
66 ± 8	59 ± 14	1.1	0	3.62 ± 0.54	1.43 ± 0.31	0.47 ± 0.21	0.79	5.8 ± 3.8	35.7 ± 6.9	13.9	2.21 ± 0.32	0.34 ± 0.11	9.3 ± 1.8
66 ± 8	51 ± 6	1.3	60	7.26 ± 0.32	0.68 ± 0.04	0.04 ± 0.01	0.95	15.7 ± 6.2	22.1 ± 3.7	41.5	5.00 ± 1.67	3.46 ± 0.87	14.5 ± 2.9

anaerobic metabolism for their total energy production.

Environmental anaerobiosis

Many molluscs use the phosphagen, arginine phosphate, as well as aspartate and glycogen for ATP production during environmental anoxia and reduce their metabolic rate considerably (see reviews by Livingstone, 1982; Gäde, 1983a). In contrast, the tail muscle of *O. limosus* utilises glycogen almost exclusively; there was no change in aspartate concentration in the tail muscle in any of the experiments.

In the foot or adductor muscle of bivalve molluscs, arginine phosphate breakdown is observed to be greatest during the first hour of anoxia (Meinardus and Gäde, 1981; Gäde, 1983b), whereas in the tail muscle of *O. limosus* transphosphorylation contributes to ATP production during the later phase of anaerobiosis. The energy charge of the tail muscle is maintained up to 12 hr of anoxia. This is in accordance with results for the isopod, *Cirolana borealis*, where the energy charge of the whole animal remains unchanged throughout 48 hr of anoxia (Skjoldal and Bakke, 1978). Since only quantitatively negligible amounts of D- and L-alanine and succinate are produced during anoxia in the tail muscle of *O. limosus*, as well as in whole mud shrimps (Zebe, 1982), L-lactate can be considered to be the sole important end product of anaerobic metabolism. The lactate pathway produces all the energy necessary to maintain the energy charge at a high level up to 12 hr. Lactate production in the abdominal muscle of *O. limosus* is approximately linear with time of anoxia up to 12 hr, reaching values of almost 20 μmol/g wet weight. Few data are available in the literature on lactate production in crustacean tissue during environmental anoxia. Zebe (1982), however, measured about 40 μmol lactate/g wet weight in the whole body of *U. pugettensis* after 24 hr of anoxia, and Pritchard and Eddy (1979) determined about 42 μmol lactate/ml hemolymph in the same species after 18–20 hr of anoxia. In *O. limosus* the lactate concentration in the hemolymph exceeded the muscle lactate level: after 16 hr of anoxia almost 60 μmol/g hemolymph was measured. Since the whole organism is anoxic it is obvious that all tissues have to rely on anaerobic metabolism and may contribute to hemolymph lactate level. Phillipps *et al.* (1977) have shown that lactate produced in the tail muscle during exercise diffused into the hemolymph. This may also be true for other tissues during anoxia. However, these authors also report that lactate concentration in the muscle was always greater than in the hemolymph, which is in contrast to our data during environmental anoxia, but in agreement with the situation in fishes, where a high tissue to blood lactate gradient has been determined (Wardle, 1978).

Helix pomatia is another invertebrate that shows higher blood levels of lactate than tissue levels during anoxia: whereas 50 mM D-lactate was found in the blood, only 15 mM D-lactate was measured in the liver (Wieser, 1981).

In *H. pomatia* mobilisation of $CaCO_3$ is adequate to "neutralise the large influx of organic acids (D-lactate and succinate) into the hemolymph and to keep the ionic budget balanced" (Wieser, 1981).

Helix pomatia has an easily available buffering capacity in the form of $CaCO_3$ in the shell and the lack of this may be the reason why exercise plaice keep a high lactate level (up to 45 mM) in muscle tissue (Wardle, 1978). In crustaceans, mobilisation of $CaCO_3$ from the carapace or other stores in order to prevent hemolymph acidosis during aerial exposure in the aquatic species *Cancer productus* and after exercise in the land crab *Cardisoma carnifex* was suggested by de Fur *et al.* (1981) and Wood and Randall (1981), respectively.

An increase in the hemolymph lactate level was also demonstrated during environmental anoxia in *C. californiensis* (to 5.9 μmol/ml in 18–20 hr; Pritchard and Eddy, 1979), and in *Carcinus maenas* (to 27.9 μmol/ml in hypoxic water 3 hr after aerobic shutdown; Burke, 1979; to 49.0 μmol/g after 16 hr of anoxia; Gäde and Carlsson, unpublished results). Thus, the ability to accumulate high hemolymph lactate levels is present in at least some species of crustacea, including *O. limosus*. Presumably, lactate diffuses into the hemolymph to facilitate further reduction of pyruvate by channelling the end product of the equilibrium reaction of lactate dehydrogenase out of the compartment. Since the $K_{m(pyruvate)}$ and $K_{m(lactate)}$ in the tail muscle of *O. limosus* are 0.055 and 38 mM, respectively (Urban, 1969), it is evident that a high level of lactate may be accumulated before the reaction is shifted to lactate oxidation. This can be seen during recovery from anoxia, which is a slow event requiring between 12 and 24 hr despite the fact that the oxygen consumption increases in many species to a level well above the resting level (Bridges and Brand, 1980). This "respiratory overshoot" has also been reported to occur in crayfish (Hiestand, 1931). Despite the high oxygen tension and hyperventilation, the hemolymph lactate levels in *O. limosus* were still elevated during the initial 3 hr of recovery, probably due to diffusion of lactate ions from different tissues into the hemolymph.

After 2 hr of recovery lactate oxidation may take place in all tissues of *O. limosus*, since no tissue-specific isoenzymes of lactate dehydrogenase were found (Urban, 1969). The protracted time course of hemolymph lactate removal was also observed in other crustaceans after hypoxia (Bridges and Brand, 1980). It may be indicative that all these species have no effective systems for oxidising lactate. In *C. destructor* Phillipps *et al.* (1977) found no evidence for gluconeogenesis from lactate in the midgut gland, gills or haemocytes. The time course for replenishment of arginine phosphate stores after environmental anoxia has not been investigated for any crustacean species. In *O. limosus* tail muscle recovery takes between 1 and 6 hr after 12 hr of environmental anoxia. This is somewhat longer than what was found in most molluscan muscles (for a review, see Ellington, 1983). During this time muscle lactate levels also decline. These data suggest that the energy for the build-up of pre-anoxic phosphagen levels is derived from aerobic metabolism.

Functional anaerobiosis

There are many reports on the changes in hemolymph lactate concentration in different crustaceans after functional anaerobiosis (burst activity, crawling, swimming). During forced activity (15–20 min) hemolymph lactate levels reached values of 5–12 mM in *Cancer magister* (McDonald *et al.*, 1979; Graham *et al.*, 1983), *Carcinus maenas* (Burke, 1979), *Callinectes sapidus* (Booth *et al.*, 1982) and *Crangon crangon* (Onnen and Zebe, 1983). The increase in hemolymph lactate levels were more pronounced when "mild" and "severe" exercise were compared in the land crab, *C. carnifex* (Wood and Randall, 1981). In the crayfish *C. destructor* 4 mM was found in the hemolymph about 50 min post-exercise during which 30 tail flips occurred over 2–4 min (Philipps *et al.*, 1977). Only one of these reports investigated the role of arginine phosphate during functional anaerobiosis (Onnen and Zebe, 1983). The contribution of arginine phosphate to ATP production is highest in *O. limosus*, when the animals are exercised submaximally for a long period (87–91% of the total production; Table 4) and about equal to the contribution from glycolysis in animals exercised maximally for short periods. The energy production is only insufficient in the first group to maintain the high energy charge of the abdominal muscle. The ATP consumption rate is very high in the animals exercised maximally for a short period and decreased considerably in the group exercised for about 59 sec with 66 tail flips (3348 vs 984 μmol ATP/hr and g wet weight). Restoration of the pre-exercise energy charge value and arginine phosphate concentration must include anaerobic processes, since muscle and hemolymph lactate levels rise during recovery, despite the high values of venous P_{O_2} in the first 30 min of recovery.

In the tail muscle of *Crangon crangon* (Onnen and Zebe, 1983), it was also shown that the phosphagen stores were rapidly utilised during work and then, when most of the phosphagen had disappeared, the energy charge dropped to about 0.6. These data agree well with our results for *O. limosus* tail muscle during heavy exercise (Table 3, line 6). However, there are also differences between the two studies: (1) in the shrimp's abdominal muscle ATP level and phosphagen reserves are both fully replenished during a 30 min period of recovery, whereas in *O. limosus* only

Table 4. Comparison of ATP equivalents (μmol ATP/g wet weight) derived from arginine phosphate and from glycolysis (lactate production) in the tail muscle of *O. limosus* during functional anaerobiosis. Calculation assumes no aerobic contributions to ATP production

Treatment	ATP equivalents			% of total ATP equivalents	
	Phosphagen	Glycolysis	Sum	Phosphagen	Glycolysis
Exercised for 20 sec with 39 tail flips	10.0	8.6	18.6	54	46
Exercised for 25 sec with 27 tail flips	10.0	1.5	11.5	87	13
Exercised for 59 sec with 66 tail flips	15.0	1.4	16.4	91	9

the pre-exercise value for the energy charge was reached after 1 hr of recovery, but arginine phosphate levels were restored only to about 70% of the control; (2) as hemolymph lactate levels after exercise plus recovery (Table 3, line 7) are five to ten times higher than controls (Table 3, line 1 and line 6) we argue that there is a removal of lactate out of the muscle into the hemolymph. This is not observed in the shrimp.

Finally, we want to point out a possible regulatory mechanism during functional anaerobiosis. Guderley *et al.* (1976) found pyruvate kinase (PK) in the tail muscle of the pelagic shrimp, *Oplophorus gracilirostris*, which could be strongly inhibited by MgATP and arginine phosphate. In our study a drop in the phosphagen already occurred during the short, heavy exercise period and, during longer, milder exercise, the ATP concentration was also diminished. Both changes could lead to a deinhibition of PK and the metabolic flux through glycolysis could increase resulting in lactate production. The inhibitory effect of arginine phosphate on PK from crude invertebrate tissue extracts, however, was questioned by de Zwaan and Ebberink (1978) due to the presence of contaminating enzyme activities in the preparations. Recently, Storey (1981) showed the inhibitory effect of arginine phosphate on different enzymes of the cephalopod *Sepia officinalis*, including PK, after separation of the enzymes by isoelectrofocusing. In *O. limosus* tail muscle the existence of an allosterically activated (by fructose-1,6-diphosphate) PK has been shown (Lesicki and Obuchowicz, 1980), resembling more the L-type PK of mammalian sources. This is in contrast to the PK of *O. gracilirostris* (Guderley *et al.*, 1976) resembling mammalian M-type PK. A re-investigation of *O. limosus* PK seems needed to establish its regulatory properties on glycolysis.

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Note added in proof

The following paper by W. R. England and J. Baldwin: "Anaerobic energy metabolism in the tail musculature of the Australian yabby *Cherax destructor* (Crustacea, Decapoda, Parastacidae). Role of phosphagens and anaerobic glycolysis during escape behaviour" has been accepted for publication in *Physiol. Zool.* and might be of interest in the context of the present paper.

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THE COURSE OF PROTEIN DIGESTION IN ATLANTIC COD (*GADUS MORHUA*)

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Abstract—1. Samples of soluble dry matter from the digestive tract of cod (*Gadus morhua*), fed a diet based on saithe fillet with and without added [^{14}C]lysine labelled fish muscle protein, were fractionated in a Sephadex G-25 gel filtration column.

2. The protein derived water soluble dry matter was mainly composed of proteins and polypeptides in the stomach, but was mainly composed of polypeptides, short peptides and free amino acids in the pyloric ceca and anterior ileum.

3. The molecular weight distribution pattern indicated predominant proteolytic activity in the stomach similar to that of pepsins, and in the pyloric ceca and anterior ileum similar to that of pancreatic proteases and peptidases.

INTRODUCTION

The presence of digestive proteases and peptidases has been demonstrated in the gastrointestinal tract of several fish species (Kapoor *et al.*, 1975; Fänge and Grove, 1979). The absorbed products of luminal digestion consist of amino acids, di- and tripeptides. The process of absorption of short peptides involves two mechanisms, entry of di- and tripeptides into the absorptive cells with intracellular hydrolysis followed by transfer of free amino acids to the blood by the usual pathways (Matthews, 1975). Brush-border peptidases have been demonstrated in the intestine of catfish (*Ameiurus nebulosus* L.) and common carp (*Cyprinus carpio* L.) by Fraisse *et al.* (1981) and in the intestine of rainbow trout (*Salmo gairdneri*) by Ash (1980). Amino acids can be absorbed more rapidly from short peptides than from the equivalent amount of free amino acids (Matthews, 1975). In the cod, dietary proteins were absorbed primarily in the anterior part of the ileum including the pyloric ceca (Lied *et al.*, 1982; Lied and Njaa, 1982). This paper presents data on the course of protein digestion in different parts of the gastrointestinal tract of cod with regard to the mol. wt distribution of solubilized protein components in the digest.

MATERIALS AND METHODS

Cod (*Gadus morhua*) were caught off the western coast of Norway. They were transferred to a sheltered 25 m^3 tank supplied with running sea water at 8°C and 35‰ S. The photoperiod was regulated to 12 hr dark and 12 hr light. The fish were acclimatized to the experimental conditions for 10 weeks. Whole capelin (*Mallotus villosus*) or sprat (*Sprattus sprattus*) were fed daily *ad lib.* during the acclimatization period. The fish were starved for 5 days prior to the experiment to ensure an empty gastrointestinal tract. Two feeding experiments were performed. In Expt 1, 12 fish ranging from 1.1 to 2.3 kg, were force-fed moist pellets 2 cm in diameter and 4–8 cm in length, consisting of 86% minced fish fillet from saithe (*Pollachius virens*), 7% capelin oil and 7% dextrin. Moist pellet measuring 4 cm in length corresponded to 16 g feed, and each fish was fed 16 g/kg body wt. Gastrointestinal contents collected approximately 36 hr after feeding were taken for analysis. Experiment 2 included ten fish ranging in weight from 0.8 to 1.1 kg. The feed, as well as the feeding and sample collection procedure, was the same as in Expt 1, but with one modification. A part of the saithe fillet used in the feed was replaced by [^{14}C]lysine labelled fish muscle protein prepared from rainbow trout. Labelled muscle protein was prepared by feeding rainbow trout, weighing approx. 50 g, for 10 days with pelleted trout feed containing [L-^{14}C(U)]lysine (spec. activity 270 mCi/m mol). Trout muscle was homogenized in a volume of two times its weight of Tris–buffer (0.05 M, pH 7.5 at 25°C) using an Ultra Turrax homogenizer and centrifuged for 10 min at 3000 *g*. Batches of the supernatant containing the water soluble proteins, were passed through a 5 × 10 cm column of Sephadex G-25, particle size 20–80 μm, in order to remove short peptides and free amino acids. The protein containing part of the supernatants was then combined with the washed residue of water insoluble proteins and mixed into minced fish fillet from saithe.

The gastrointestinal tract was divided into segments by ligations at the oesophagus, immediately before the pyloric ceca, at the first cranial, the first caudal, the second cranial bend of the ileum and at the ileorectal valve. The five segments were named stomach, pyloric ceca, anterior ileum, middle ileum and posterior ileum. The rectum was not included. The contents of the segments were washed into containers with 0.9% NaCl solution, frozen immediately and freeze-dried. Analyses were carried out on pooled samples prepared by mixing equal amounts of homogenized dry matter from corresponding segments. Pooled freeze-dried gastrointestinal samples were then suspended in distilled water (1 g–10 ml), soaked for 10 min and centrifuged for 50 min at 10,000 *g* at 4°C. A portion of the supernatant was collected and freeze-dried. Prior to gel filtration, samples of soluble intestinal dry matter were dissolved in buffer and filtered through a 0.45 μm membrane filter. The fractionation was carried out at 4°C on a 1.6 × 90 cm column using Sephadex gel filtration medium, type G-25, particle size 20–80 μm (Pharmacia). The column, which had a void volume of 72 ml, was calibrated with cytochrome C (mol. wt 12,400), insulin B chain (mol. wt 3484), polymyxin B sulphate (mol. wt 1447), L-methionyl-L-alanyl-L-serine (mol. wt 307) and a mixture of 18 amino acids. Portions of 20 mg (Expt 1) or 100–140 mg (Expt 2) of water soluble dry matter dissolved in 5 ml buffer were applied to the column and eluated at a rate of 57 ml/hr with 0.02 M sodium phosphate

buffer (pH 7.6) containing 0.1 M sodium chloride. Soluble proteins and polypeptides of mol. wt above 4000 were eluted between 70 and 85 ml, polypeptides of mol. wt between 4000 and 300 were eluted between 85 and 140 ml, and short peptides and free amino acids between 140 and 190 ml. Fractions of 2 (Expt 1) and 4 (Expt 2) ml were collected.

When samples from Expt 1 were analysed for α-amino-N 0.5 ml of each fraction was heated overnight with 0.5 ml of conc. HCl at 120° C. To each hydrolysate was added 1.2 ml of 4 N NaOH and 0.5 ml of 4 N sodium acetate buffer (pH 5.5), and made up to 5 ml with distilled water. α-Amino-N was estimated by the ninhydrin reaction. A standard curve was prepared with graded concentrations of leucine, and the results were expressed in terms of "leucine-equivalent".

When samples from Expt 2 were analysed for [^{14}C]lysine activity, 1 ml of each fraction was assayed for radioactivity in a liquid scintillation counter at 60% efficiency, using 10 ml of Biofluor (NEN Chemicals).

RESULTS AND DISCUSSION

Solubilization of the feed and proteolysis of dietary proteins precede its absorption in the gastrointestinal tract. In Expt 1 the fractions of water soluble dry matter and α-amino-N increased from 8 and 2% in the feed, to 29 and 8% of the total dry matter, respectively, in the gastric content sampled 36 hr after feeding (Table 1). Gel filtration showed that two thirds of the soluble α-amino-N in the feed was in the form of short perptides and free amino acids (Table 1). In the stomach the α-amino-N fraction was composed mainly of dissolved proteins and polypeptides, which constituted 53% of the solubilised α-amino-N concentration. The concentration of short peptides was relatively low as 17% of the α-amino-N was of mol. wt <300. The chyme was further solubilized in the pyloric ceca region showing soluble dry matter and α-amino-N fractions of 66 and 18% of the total dry matter respectively (Table 1). As compared with that of the gastric content the mol. wt distribution of the α-amino-N shifted towards an increased fraction of free amino acids and short peptides, and a decreased fraction of polypeptides of mol. wt of 4000–300, each fraction constituting 31 and 36%, respectively, of the total solubilized α-amino-N. As in the stomach, the relative amount of protein was approximately 30% of the total amount of soluble α-amino-N. The further passage of chyme into the anterior ileum decreased the fractions of soluble dry matter and α-amino-N to 39 and 6% of the total dry matter, respectively, due to the absorption of feed components in the pyloric ceca. Previous cod studies have shown that 91% of the protein (N × 6.25) and 96% of the amino acid absorption (Lied *et al.*, 1982; Lied and Njaa, 1982) and 82% of the fat absorption (Lied and Lambertsen, 1982) takes place in the pyloric ceca and anterior ileal region of the digestive tract. In the anterior ileum polypeptides amounted to 38% while short peptides and free amino acids amounted to 44% of the soluble α-amino-N. Only 17% was made up by proteins.

Determination of soluble α-amino-N in the gastrointestinal tract by the ninhydrin reaction includes α-amino-N of endogenous origin. The possible effect of endogenous α-amino-N on the mol. wt distribution pattern of proteins, peptides and amino acids in the soluble fraction of the gastrointestinal content was avoided by feeding cod a diet containing [^{14}C]lysine labelled fish muscle protein (Expt 2). Equal amounts of pooled samples of the soluble dry matter collected from the gastrointestinal tract of cod 36 hr after feeding were fractionated by gel filtration. The profiles of isotope activities are shown in Fig. 1 and the percentage distribution of the isotope activity in relation to mol. wt groups is given in Table 2. The protein fraction made up 17–24% of the total isotope activity in the soluble dry matter in all gastrointestinal segments. In the stomach the major part of the isotope activity was found in the fractions containing polypeptides, while only 11% of the lysine was present in short peptides and as the free amino acid. As chyme was transported towards the rectum the relative composition of isotope containing components shifted towards decreasing amounts of peptides of mol. wt between 4000 and 300, and increasing amounts of free amino acids and peptides of mol. wt of less than 300. Thus in the posterior ileum 22% of the [^{14}C]lysine was found in the fractions containing polypeptides while 60% of the amino acid was present in the form of short peptides and as the free amino acid. As shown in Fig. 1 the total isotope activity given as c.p.m./g soluble dry matter were high in the pyloric ceca and anterior ileal region but was followed by a progressive decrease in the succeeding intestinal segments. This result indicates that amino acids are absorbed along the total length of the intestinal tract in cod.

The presence of digestive proteases as pepsin, chymotrypsin, trypsin, carboxypeptidases, elastase, di- and tripeptidases has been demonstrated in the gastrointestinal tract of several fish species (Kapor *et al.*, 1975; Fänge and Grove, 1979). The composition of proteins, peptides and free amino acids in the water soluble content are indicative of the enzymatic activity in the various parts of the gastrointestinal tract. Peptic digestion yields polypeptides of very diverse sizes, but few amino acids. The large fraction

Table 1. Soluble dry matter, soluble α-amino-N and the percentage mol. wt distribution of the soluble α-amino-N concerning proteins (mol. wt >4000), polypeptides (mol. wt 4000–300) and short peptides and free amino acids (mol. wt <300). Expt 1.

Sample from	Soluble		Mol. wt distribution		
	Dry matter (mg/g)	Leucine equivalents (mg/g)	>4000 (%)	4000–300 (%)	<300 (%)
Feed	84	20	8.2	24.1	67.7
Stomach	294	86	29.2	53.1	17.7
Pyloric ceca	661	177	32.3	31.0	36.7
Anterior ileum	398	61	17.0	38.8	44.2

Soluble dry matter is given as mg/g total dry matter in the samples. Soluble α-amino-N is given as mg leucine-equivalents/g total dry matter in the samples.

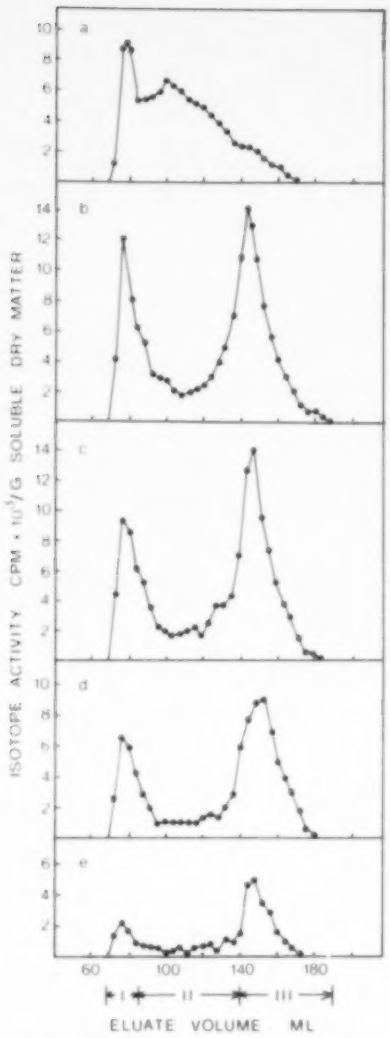

Fig. 1. Fractionation in Sephadex G-25 of [^{14}C]lysine labelled soluble dry matter in the stomach (a), pyloric ceca (b), anterior ileum (c), middle ileum (d) and posterior ileum (e) of cod fed a diet containing [^{14}C]lysine labelled fish muscle protein (Expt 2). Soluble proteins and polypeptides of mol. wt greater than about 4000 were eluted between 70 and 85 ml (I), polypeptides of mol. wt between 4000 and 300 were eluted between 85 and 140 ml (II) and short peptides and free amino acids were euluted between 140 and 190 ml (III).

of polypeptides and small fraction of short peptides and free amino acids found in the stomach of cod indicates a predominant proteolytic activity similar to that of pepsins.

The presence in cod of pancreatic proteases and peptidases has been demonstrated in the mesentery of pyloric ceca (Overnell, 1973). The mesentery contains numerous small pancreatic ducts, which converge into a few larger ones entering the ileum just anterior to the bile duct (Bishop and Odense, 1966). By the action of pancreatic proteases, polypeptides are converted into short peptides and free amino acids. In

Table 2. The distribution of [^{14}C]lysine activity in proteins (mol. wt >4000), polypeptides (mol. wt 4000–300) and in short peptides and as free amino acid (mol. wt <300) in samples of soluble dry matter from the gastrointestinal tract of fish from Expt 2

Sample from	Mol. wt distribution		
	>4000 (%)	4000–300 (%)	<300 (%)
Feed	100.0	—	—
Stomach	24.5	64.4	11.1
Pyloric ceca	20.4	40.4	39.6
Anterior ileum	23.0	33.8	43.2
Middle ileum	20.9	24.0	54.1
Posterior ileum	17.8	21.9	60.3

The values are given as percentage of total isotope activity in the soluble dry matter.

Expt 1, 36 and 44%, respectively, of the water soluble α-amino-N fraction found in the pyloric ceca and anterior ileum were made up by short peptides and free amino acids. Correspondingly, in Expt 2 39 and 43%, respectively, of the total isotope activity in soluble dry matter was found in the fractions containing short peptides and free amino acid. Thus, on the passage of digests from the stomach to the pyloric ceca and ileum in cod, further proteolysis occurs through the action of enzymes with proteolytic activity similar to that of pancreatic proteases and peptidases.

Recent studies with atlantic cod have shown that feeding short-time starved fish stimulated, within 5–6 hr, excretion of exogenous ammonia into the water (Lied and Braaten, 1984), and further stimulated the protein synthesis in the white trunk muscle within 4 hr (Lied *et al.*, 1983). This suggests a rapid digestion and absorption of feed proteins. Amino acids are absorbed more rapidly from short peptides than from the equivalent amount of free amino acids (Matthews, 1975). The cod possesses about 300 pyloric ceca (Bishop and Odense, 1966), which form a large structure around the ileum just posterior to the stomach. The solubilization luminal content of the pyloric ceca was rich in short peptides and free amino acids. Thus, absorption of short peptides in the pyloric ceca will increase the efficiency of amino acid absorption leading to a rapid flow of amino acids into the amino acid pool.

Austreng (1978) and Dabrowski and Dabrowska (1981), reported that protein and amino acids were absorbed in the stomach of rainbow trout. In the present study with cod, the concentration of short peptides and free amino acids in the solubilized gastric content was low. Unless cod stomach possesses the ability to absorb polypeptides, the gastric absorption of amino acids may be considered negligible. However, the possibility of gastric absorption of peptides and amino acids may not be rejected.

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ROLE OF OUABAIN AND DIURETICS ON SODIUM, POTASSIUM AND CHLORIDE RETENTION IN PERFUSED RAT KIDNEY

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Abstract—1. A novel *in situ* kidney perfusion technique is described in Sprague–Dawley rats. The procedure involves retrograde perfusion from the renal veins via the kidneys, and then through the renal arteries and dorsal aorta.

2. Ouabain (15 mM) in perfusate increased Na retention by 92%, decreased K retention by 53% and produced no effect on Cl retention.

3. Ethacrynic acid (1 mM) in perfusate decreased Na retention by 52%, increased K retention by 105% and decreased Cl retention by 27%.

4. Furosemide (1.5 mM) in perfusate decreased Na retention by 52%, increased K retention by 47% and decreased Cl retention by 56%.

5. The Na–K–ATPase pump localized at the peritubular side of the proximal tubule cell is ouabain sensitive and Mg dependent.

6. An Na–K pump responsible for Na influx and K efflux exists at the luminal side of the proximal tubule cell and is ethacrynic acid and furosemide sensitive.

INTRODUCTION

The processes controlling Na, K and Cl distribution in kidneys have been the subject of extensive investigation. Investigators have reported results on Na, K and Cl filtration and reabsorption using a variety of techniques and methods. These techniques include perfusion experimentation with whole kidney (Swanson *et al.*, 1956; Hoshiko *et al.*, 1956; Strickler and Kessler, 1963; Nishitsutsuji-Uwo *et al.*, 1967; Torretti *et al.*, 1972; Wilkinson *et al.*, 1979), experimentation with kidney slices (Mudge, 1951; Whittam and Davies, 1953; Kleinzeller and Knotkova, 1964; Smyth, 1966; Wald *et al.*, 1977), experimentation involving micropuncture of different segments of the nephron (Richards and Walker, 1937; Walker and Oliver, 1941; Lewy and Windhager, 1968; Jamieson, 1970), and finally experiments utilizing microelectrodes in intracellular measurements (Khuri, 1980).

This manuscript describes a novel *in situ* kidney perfusion technique in the rat with emphasis on its applicability in the study of kidney functions. The role of metabolic inhibitors and diuretics on Na, K and Cl distribution in the perfused kidneys are reported. A physical model which describes the mechanisms controlling the distribution of ions across kidney cells is proposed.

MATERIALS AND METHODS

Protein determination

The method of Jackson and McCandless (1978) is used for protein determination and is described as follows. To 1.1 ml of the protein sample or tissue homogenate, 1.2 ml of 8% trichloroacetic acid solution and 0.5 ml of 0.02% agarose (Fluka AG, Buchs, Switzerland) solution are added. The mixture is made to stand for 40 min until turbidity is maximally developed and analysed spectrophotometrically at 500 nm. A standard curve is prepared using standard solutions of bovine serum albumin (Fluka AG, Buchs, Switzerland) containing 5, 12.5, 25 and 50 mg per 100 ml Krebs improved Ringer solution (Dawson *et al.*, 1959).

Preparation of animal

Adult female Sprague–Dawley rats, *Rattus norvegicus*, weighing 150–200 g are fed *ad libitum* and anesthetized by i.p. injection of 80 mg/kg of 5-s-butyl-5-ethyl-2-thiobarbituric acid sodium salt (Inactin®, BYK Gulden, Konstanz, W. Germany). The animal is then shaved on the abdomen and neck regions and the ventral side of the neck is longitudinally opened and the animal tracheotomized. The left carotid artery is exposed and catheterized for later manipulation in the experiment.

A mid-line incision is made from the thoracic cavity to the groin region, and the intestines are gently lifted from the abdominal cavity so as to expose organs and viscera needed in the experiment. The right ovarian vein is ligated and the aorta and vena cava are isolated and exposed below the ileo-lumbar region. A loose ligation is made around the aorta and vena cava in preparation for a tighter ligation later on in the experiment. The right (or left) ileo-lumbar vein is catheterized with polyethylene tubing (i.d. 0.28 mm, o.d. 0.6 mm) connected to a 50 ml all-glass syringe attached to an infusion pump (Unita I, B. Braun, Melsunger, W. Germany). Perfusion of both kidneys is maintained at 5 ml·min^{-1} throughout all experiments. Figure 1 shows a sketch of the renal system of the female rat with principal blood vessels in the posterior region. The sketch shows the perfusion route (right ileo-lumbar vein → posterior vena cava → left and right renal veins → left and right renal arteries → dorsal aorta → carotid artery). All other arteries and veins leading to and from this route were ligated to prevent leakage of perfusate.

Perfusion of kidneys

There were three major steps involved in each perfusion

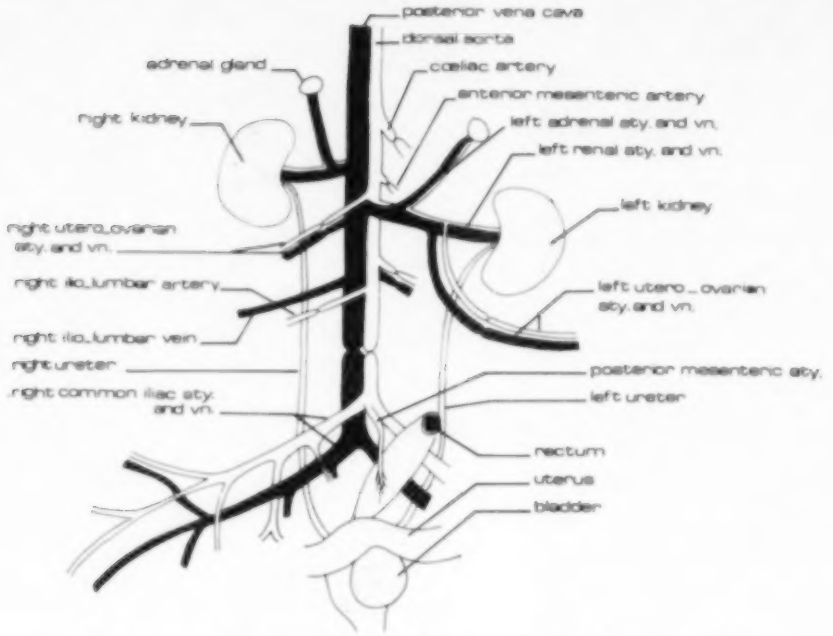

Fig. 1. Sketch of the renal system of the female rat with principal blood vessels.

experiment. Firstly, 15 ml of oxygenated (gassed with 95% O_2/5% CO_2) Krebs improved Ringer solution containing 100 units of sodium heparin was infused to clear the kidneys from blood. At this stage the aorta and vena cava were ligated. Secondly, 20 ml of oxygenated buffer containing the desired amounts of radiolabelled Na, K or Cl (1 μCi ^{22}Na, 2.5 μCi ^{42}K, or 50 μCi ^{36}Cl) were perfused. This step also included the addition of ouabain (15 mM in buffer) and ethacrynic acid (1 mM in buffer) to the perfusate whenever their effect was to be investigated. Thirdly, 15 ml of oxygenated buffer was infused for a final clearing of any radioactivity retained in the vascular supply of the kidneys.

In all the above-mentioned steps, the perfusates were infused at a rate of 5 ml min^{-1} through the right ileo-lumbar vein, and collected at a rate of $\cong$4.6 ml min^{-1} through the carotid artery. The kidneys were then excised from the animal, washed well and cleared from connective tissues and fat material. They were then carefully decapsulated, blotted on filter paper to remove traces of adsorbed water and each accurately weighed on an analytical balance.

Measurement of Na *and* K *retention*

The decapsulated kidneys were finally homogenized with 10 ml buffer, and then analytically transferred and made up to volume in a 25 ml volumetric flask. Homogenate, measuring 5 ml, was pipetted into 10 ml screw-cap test tubes for γ-counting, and the remainder were used for protein analysis. In order to calculate total Na and K retained in kidneys, the ratio of ^{22}Na/^{23}Na in buffer and ^{42}K/^{39}K in buffer was measured, and then from the specific activities and disintegration counts of the labelled ions in the tissue, the total amount of Na or K per mg protein was estimated.

Measurement of Cl *retention*

The procedure reported above was employed. However, 1 ml of the homogenate was pipetted and transferred into 10 ml liquid scintillation vial and the tissue was allowed to be digested with 1 ml hyamine hydroxide for 12 hr. To this 5 ml of Bray's solution was added, and the mixture was assayed for ^{36}Cl in a liquid scintillation spectrometer. The amount of Cl retained by the kidneys was estimated by a procedure similar to that for Na and K.

RESULTS

Choice of perfusion time

The renal outflow of the perfusate through the carotid artery (5 ml min^{-1}) matches the normal arterial renal plasma flow in rat kidneys (5–7 ml min^{-1}) (Ackermann, 1978), and thus simulated physiologic conditions, although the perfusion was in retrograde. It was observed that if the total perfusion time exceeded 10 min (time needed to perform the three perfusion steps per experiment), then the viability of the kidneys became questionable. Inconsistent data were observed with experiments whose timing exceeded 20 min.

Effect of ouabain on Na, K *and* Cl *retention*

Table 1 shows the results for the retention of the three ions. Ouabain (15 mM in buffer) almost doubled the amount of Na retained (4.72 ± 0.49 vs 9.06 ± 0.32 μg Na per mg protein); however, the amount of K retained was almost half that of the control ($19.0 \pm 3.9 \times 10^{-5}$ vs $9.0 \pm 0.85 \times 10^{-5}$ μg K per mg protein). Chloride retention increased significantly when 15 mM ouabain was perfused with the buffer (0.125 ± 0.003 vs 0.143 ± 0.005 μg Cl per mg protein).

Effect of ethacrynic acid on Na, K *and* Cl *retention*

The effect of 1 mM concentration of ethacrynic acid on Na, K and Cl retention is shown in Table 2. Sodium retention decreased significantly in the presence of ethacrynic acid (4.72 ± 0.49 vs 2.27 ± 0.18 μg Na per mg protein), while K retention increased ($19.0 \pm 3.90 \times 10^{-5}$ vs $39.0 \pm 7.0 \times 10^{-5}$ μg Na per

Table 1. Effect of ouabain on Na, K and Cl retention in perfused rat kidney

Solution	Na retention (μg Na/mg protein) ±SEM	K retention (μg K/mg protein) $\times 10^{-5}$ ± SEM	Cl retention (μg Cl/mg protein) ±SEM
Krebs Ringer (control)	4.72 ± 0.49[a]	19.00 ± 3.90[c]	0.125 ± 0.003[e]
Krebs Ringer + 15 mM ouabain	9.06 ± 0.32[b]	9.00 ± 0.85[d]	0.143 ± 0.005[f]

Significant difference ($N = 6$).
$P < 0.001$ (a,b); (c,d); (e,f).

Table 2. Effect of ethacrynic acid on Na, K and Cl retention in perfused rat kidney

Solution	Na retention (μg Na/mg protein) ±SEM	K retention (μg k/mg protein) $\times 10^{-5}$ ± SEM	Cl retention (μg Cl/mg protein) ±SEM
Krebs Ringer (control)	4.72 ± 0.49[a]	19.00 ± 3.90[c]	0.125 ± 0.003[e]
Krebs Ringer + 1 mM ethacrynic acid	2.27 ± 0.18[b]	39.00 ± 7.00[d]	0.091 ± 0.23[f]

Significant difference ($N = 6$).
$P < 0.001$ (a,b); (c,d); (e,f).

mg protein). Chloride retention decreased when 1 mM ethacrynic acid was added to the perfusate (0.125 ± 0.003 vs 0.091 ± 0.023 μg Cl per mg protein).

Effect of buffer solution containing ouabain and ethacrynic acid on sodium retention

Table 3 shows the data on the effect of a mixed solution of 15 mM ouabain and 1 mM ethacrynic on Na retention. When both substances are present, decrease in Na retention was observed when compared to control and to when ouabain alone is mixed with the buffer. In the latter two cases, the lowering of Na retention did not reach that of when ethacrynic acid was present alone in the buffer.

Buffer of Mg*-free solution on* Na *retention*

The results of perfusing a Mg-free buffer on Na retention is shown in Table 3. A statistically significant increase in Na retention was observed when the buffer lacks Mg.

Effect of furosemide on Na, K *and* Cl *retention*

Table 4 shows that both furosemide and ethacrynic acid act similarly on the mechanisms controlling Na, K and Cl retention, while ouabain had resulted in the opposite effects.

Effect of perfusing K*-free Krebs Ringer with or without ethacrynic acid and/or ouabain on* Na *retention*

The results are shown in Table 5. Perfusing with K-free buffer, Na retention increased when compared to control. The presence of 1 mM ethacrynic acid in the K-free buffer resulted in a decrease in Na retention below control values. However, the results did not significantly differ from those of the Krebs Ringer containing 15 mM ouabain and 1 mM ethacrynic acid.

Effect of K *on* Na *retention*

The absence of K in the perfusate resulted in increase in Na retention (Table 6). The gradual addition of K to the K-free buffer resulted in a decrease

Table 3. Effect of ouabain, ethacrynic acid and Mg-free Krebs Ringer on Na retention in whole kidney

Solution	Na retention (μg Na/mg protein) ± SEM
Krebs Ringer (control)	4.72 ± 0.49[a]
Krebs Ringer + 15 mM ouabain	9.06 ± 0.32[c]
Krebs Ringer + 1 mM ethacrynic acid	2.27 ± 0.18[b]
Krebs Ringer + 1 mM ethacrynic acid + 15 mM ouabain	3.70 ± 0.28[d]
Mg-free Krebs Ringer	6.58 ± 0.62[e]

Significant difference ($N = 6$).
$P < 0.001$ (a,b); (a,c); (a,d); (b,c); (b,d); (c,d); (a,e).

Table 4. Comparative effect of ouabain, ethacrynic acid and furosemide on Na, K and Cl retention

Solution	Na retention (μg Na/mg protein) ±SEM	K retention (μg K/mg protein) $\times 10^{-5}$ ± SEM	Cl retention (μg Cl/mg protein) ±SEM
Krebs Ringer (control)	4.72 ± 0.49[a]	19.00 ± 3.90[e]	0.125 ± 0.003[i]
Krebs Ringer + 15 mM ouabain	9.06 ± 0.32[b]	9.00 ± 0.85[f]	0.143 ± 0.005[j]
Krebs Ringer + 1 mM ethacrynic acid	2.27 ± 0.18[c]	39.00 ± 7.00[g]	0.091 ± 0.023[k]
Krebs Ringer + 1.5 mM furosemide	2.27 ± 0.12[d]	28.00 ± 1.70[h]	0.055 ± 0.004[l]

Significant difference ($N = 6$).
$P < 0.001$ (a,b); (a,d); (a,c); (b,d); (b,c); (e,f); (e,h); (e,g); (f,h); (g,h); (i,j); (i,k); (j,l); (j,k).
$0.4 < P < 0.5$ (c,d).
$0.3 < P < 0.4$ (i,j).

Table 5. Effect of K-free solution with or without ethacrynic acid and/or ouabain on Na retention

Solution	Na retention (μg Na/mg protein) ± SEM
Krebs Ringer (control)	4.72 ± 0.49[a]
K-free Krebs Ringer	6.71 ± 0.40[b]
K-free Krebs Ringer + 1 mM ethacrynic acid	3.84 ± 0.11[c]
Krebs Ringer + 15 mM ouabain + 1 mM ethacrynic acid	3.70 ± 0.28[d]

Significant difference ($N = 6$).
$P < 0.001$ (a,b); (a,c); (a,d); (b,c).
$0.3 < P < 0.4$ (c,d).

Table 6. Effect of adding K to K-free Krebs Ringer on Na retention

Solution	Na retention (μg Na/mg protein) ± SEM
K-free Krebs Ringer	6.71 ± 0.40[a]
K-free Krebs Ringer + 0.5 mM K	5.26 ± 0.37[b]
+ 1.0 mM K	4.99 ± 0.26[c]
+ 2.0 mM K	4.91 ± 0.17[d]
+ 3.0 mM K	4.76 ± 0.11[e]
Krebs Ringer (control) (containing 7.7 mM K)	4.72 ± 0.49

Significant difference ($N = 6$).
$P < 0.001$ (a,b); (a,d).
$0.02 < P < 0.05$ (b,d).
$0.4 < P < 0.5$ (c,d).
$0.05 < P < 0.1$ (c,e).

in Na retention, and at K concentration > 0.5 mM, Na retention decreased to the level of controls (7.7 mM K).

DISCUSSION

The mechanisms involved in renal ionic transport, binding or retention may be controlled by active or passive processes depending on whether they are functionally related to a pump activity or mainly due to simple membrane binding or free diffusion. Biochemical studies on renal tissues have so far not shown the presence of any active pump mechanism other than the Na–K–ATPase located exclusively on the basal–lateral side of the tubular cells (Ernst, 1975). A Mg-dependent ATPase pump exists in the renal tissues and is inhibited by ethacrynic acid, while the Na–K–ATPase pump is only partially inhibited (Whittembury, 1971). A non-electrogenic Na : H exchange pump exists across the brush border membrane of renal tubules (Sachs, 1977). Therefore, the Na gradient may be only partially controlled by the Na–K–ATPase pump, and the remainder by other neutral or electrogenic pumps such as Mg-dependent ATPase, HCO_3 : Na cotransport pump, Na : H counter transport pump, etc. Khuri (1980) suggested a scheme showing possible active pumps and passive processes across the necturus proximal tubule cell. The model (Fig. 2) shows the Na–K–ATPase to be the only active pump at the peritubular side, while the luminal side holds three other Na pumps, namely, a Na–Glucose cotransport pump directed inwards, a Na–Cl cotransport pump also directed inwards, and a Na–H counter transport pump responsible partially for Na influx and H efflux.

The data reported in this paper support the model proposed by Khuri (1980). Ouabain had a localized effect on the peritubular side and thus causing increase in internal Na. Therefore, ouabain does not necessarily transverse the peritubular side of the proximal tubule cell and then diffuse to the luminal side to exert its effect. However, the data of Table 3 shows that the Na–K pump present at the peritubular side is Mg-dependent which is contrary to what has been reported by Whittembury (1971). Diuretics such as ethacrynic acid and furosemide act through different mechanisms and have no competitive effect with ouabain on the ouabain-sensitive pump (Table 4). Therefore, if the diuretics are only acting at the peritubular side, and if they exert little or no effect on the ouabain-sensitive pump, then their action should be essentially on an Na–K counter transport pump with Na directed inwards and K outwards. As Na influx decreases, Na and water retention in the interstitium and possibly in the intracapillary lumen would increase. This could possibly lead to edema rather than to diuresis. Therefore, it can be concluded that the ethacrynic acid and furosemide-sensitive pump is located at the luminal side. During the perfusion experiments, the diuretics initially pass in the proximity of the peritubular side, and then get translocated to the luminal side where they exert their effect. The proposed ethacrynic acid and furosemide-sensitive pump could be responsible for Na influx and K efflux (Fig. 2).

The results of Tables 5 and 6 show that when K-free buffer was perfused with ethacrynic acid, a nearly identical Na retention effect was observed as when the Krebs improved Ringer was simultaneously perfused with ouabain and ethacrynic acid. Thus, in the absence of K, the major Na–K pump at the peritubular side is inactive and the presence of ethacrynic acid did not drastically change the effect of ouabain. In conclusion,

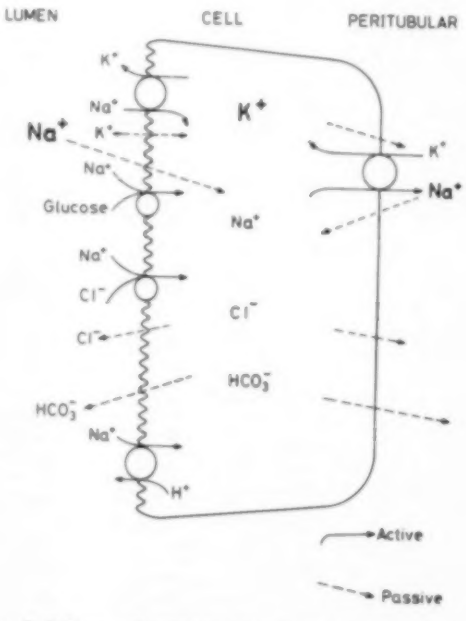

Fig. 2. Schema of proximal tubule cell incorporating all mechanisms responsible for ionic distribution under steady-state and induced transient changes.

the major Na–K pump at the peritubular side is irreversible after inhibition with ouabain because even though ouabain is washed out or replaced with ethacrynic acid, the amount of Na retained was less than control values and maximum Na inhibition was observed.

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THERMAL PREFERENCE AND TEMPORAL DISTRIBUTION IN THREE CRAYFISH SPECIES

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Abstract—1. Acute thermal preference and final preferendum were determined for three species of crayfish. In all three species the final preferenda nearly matched ambient temperature encountered during months of maximum activity. *Procambarus spiculifer* also exhibited a circadian cycle in its acute preference, matching habitat temperature changes in both magnitude and timing. *P. clarkii* exhibited a similar cycle but of smaller amplitude.

2. *Cambarus latimanus* are not active on a daily basis and did not show the circadian cycle.

3. The acute preference of *P. clarkii* was lower when they were held in deep water as compared to those held in shallow (≤ 5 cm) water.

4. It is postulated that these measures of thermal preference do not function for thermal regulation but rather serve as cues for arousal from either hibernation or their daily refugia.

INTRODUCTION

Both marine and freshwater crustaceans have routinely been shown to possess distinct thermal preferences (Hoberg, 1975; Crawshaw, 1977; Tsurnamal, 1978; Pasto, 1982). It has been postulated that changes in the preferred temperatures, over either daily, tidal or seasonal cycles, are correlated with thermal heterogeneities in the animal's habitat, thus enabling them to behaviorally thermoregulate. Streams, unlike thermally stratified lakes, show little or no stratification (Williams, 1981); thus the only temperature gradient available to lotic animals exists at the confluence of cool, shaded, low order streams and warmer more open larger streams, or between surface water and burrow temperatures. Deep burrows probably offer a fairly large temperature differential, although the same situation may not exist in more typical shallow burrows (Hewlett and Fortson, 1982). This study addressed the question of whether stream crayfish that live in a nearly thermally homogeneous environment show a thermal preference.

Thermal preference studies have utilized two different measures: (1) acute thermal preference, that is strongly influenced by previous thermal acclimation (Reynolds and Casterlin, 1979); and (2) final preferendum. The latter has been defined by Fry (1947) as a "temperature around which all individuals will ultimately congregate, regardless of their thermal experience before being placed in the gradient" and "that temperature at which the preferred temperature is equal to the acclimation temperature". Many studies have indicated that the final preferendum is generally species specific and, in fish, roughly reflect their distribution (Neill, 1979), although, in some cases the final preferendum changes with environmental factors (Calhoun *et al.*, 1982). In fish species with very broad distributions the final preferendum is surprisingly constant (Beitinger and Fitzpatrick, 1979). Dawson (1975) reviewed the meaning of the final preferendum in reptiles and found that, with some exceptions, species survival was dependent on periodic attainment of their preferred range.

Crayfish studies have implicated thermal preference with seasonal migrations (Aiken, 1968; Momot and Gowing, 1972; Crawshaw, 1977) and circadian cycles (Crawshaw, 1974). In the laboratory, acclimation temperature was shown to have either a poor correlation with crayfish acute preference (Hall *et al.*, 1978; Mathur *et al.*, 1982) or no correlation (Crawshaw, 1977). Loring and Hill (1976) found that crayfish in a thermal gradient would not leave their preferred temperature to utilize shelter in less preferred temperatures; however, no predators were present during the tests. Crayfish that were injected with killed bacteria developed a behavioral fever, defined as an increase in their preferred temperature (Casterlin and Reynolds, 1977), and at least the warm receptors have been localized in the basal segments of their antennae although the cold receptors, or even their existence, could not be established (Pasto, 1982).

In the present study, I found that both the acute preference and final preferendum of three crayfish species primarily represented the temperature that the animals encounter during their daily or seasonal activity cycles. It appears that temperature choice does not function in temperature regulation except in the very broadest sense, but instead serves as an error detection system involved in behavioral arousal during the correct time of the day or year.

MATERIALS AND METHODS

Apparatus

The thermal gradient was a horizontal, linear type composed of eight chambers. Each chamber was separated by a partition that extended to within 4.5 cm of the floor, eliminating corners in which a crayfish could hide. Water in each chamber was agitated vigorously with an airstone. Warm water was pumped from a reservoir into one end chamber and cold water, from a separate reservoir, into the opposite end chamber. The outflows were from the same two end chambers. Each chamber was 12.5 cm by 12 cm and 25 cm

deep. Gradients with a temperature differential of 25–30°C were routinely maintained for days.

The entire apparatus was leveled, enclosed in a frame, and covered with black plastic sheeting. A 122 cm fluorescent light was suspended above the gradient and driven by a timer on a 12:12 light:dark cycle. Controls were run with the gradient at a constant temperature. They indicated a moderate but equal end effect and a nearly constant distribution in the six remaining chambers. The location of the crayfish was monitored by slightly pushing aside a second plastic sheet that allowed its location to be noted. The crayfish position was recorded every 5 min for a minimum of 20 observations. The temperature of the occupied chamber was monitored through a switching system and a YSI 42SF tele-thermometer and chart recorder. The determination of final preferendum involved allowing the crayfish to remain in the gradient for 24–72 hr. After each 24 hr period the temperature choice was noted and the gradient allowed to shift over a period of 30 min. If the crayfish followed the same temperature in the shifting gradient to a new chamber, that temperature was taken as its final preferendum. If they did not follow the same temperature, the animal was allowed to remain for one or two additional 24 hr periods. Seventy-two hours was the maximum time allowed. Animals were added to a chamber that was close to the ambient temperature, followed by a 30 min acclimation period before data collection began. Only one animal was used during each determination.

Specimens

Procambarus spiculifer and *Cambarus latimanus* were collected around Athens, Georgia and *Procambarus clarkii* was obtained from a commercial supplier in Louisiana. Animals were held in the laboratory at a temperature of 20–21°C, under a 12:12 (L:D) cycle, for at least 6 months. They were fed weekly on a diet of commercial shrimp pellets, composted leaves or *Elodea*. Preferendum determinations were made during all months of the year, and no attempt was made to study acclimation.

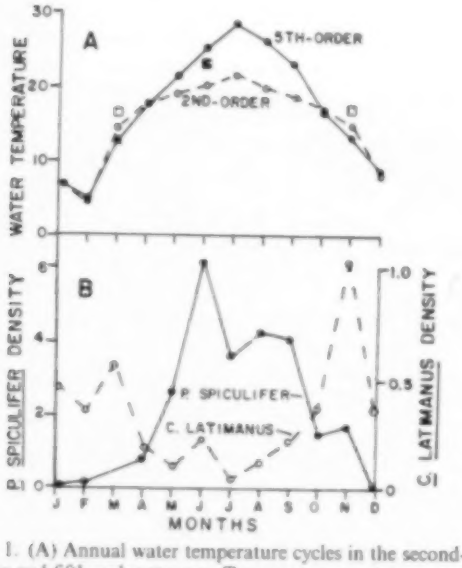

Fig. 1. (A) Annual water temperature cycles in the second-order and fifth-order streams. Temperatures were averaged for 1980–1982. (B) Annual densities of *Procambarus spiculifer* (solid line) and *Cambarus latimanus* (dashed line) for the second-order stream. Very few *C. latimanus* were found in the fifth-order stream. The solid square represents the final preferendum of *P. spiculifer* and the open squares the same for *C. latimanus*.

A full description of the field sites and sampling technique is in Taylor (1983). Briefly, crayfish were sampled using baited minnow traps in a fifth-order and two second-order sites in the same drainage. They were marked using pleural clips. Monthly samples have been taken since 1979. The stream temperatures are mid-day measurements. Monthly values were averaged over 3 years.

RESULTS

Spatial-temporal variations

The fifth-order study site was significantly warmer ($P < 0.01$) than the second-order sites in June, July, August and September (Fig. 1). No significant differences existed during the remainder of the year ($P > 0.05$), although the rate of change was different in the second-order due to the addition or loss of shade from the riparian vegetation. The magnitude of the shade effect was illustrated between 14 October and 28 November in the second-order site. The mean temperature was 15.72°C and the slope of the regression of its temperature vs time was −0.01, almost no temperature change. Apparently, radiant heating increased, due to leaf fall, at a rate nearly equal to the decrease in temperature over that 6 week period. A similar but less pronounced plateau was observed in the spring. The fifth-order did not exhibit either a spring or a fall plateau. Also, as a result of the shade, the second-order had slightly lower accumulated degree days (5575) than did the fifth-order (6187).

The two species occur at different times in the habitat, with *Procambarus spiculifer* found in maximum numbers in spring and summer and *Cambarus latimanus* in the fall with a lesser peak in the early spring (Fig. 1). Spatially *C. latimanus* is found predominantly in the lower order streams, whereas *P. spiculifer* is found in both, although at a somewhat higher density in the fifth-order (Taylor, 1983).

Thermal preference

Individual variance in thermal preference was determined by repeatedly measuring the acute preference of the same animals. Two *Procambarus spiculifer* and two *Cambarus latimanus* were tested a total of 34 times and six different *P. clarkii* were tested 55 times (Table 1). Standard deviations ranged from 2.56 to 0.70. Neither the average species final preferendum (Table 1) nor the individual's final preferendum (Table 2) showed a tight relationship to the acute preference.

Table 1. Mean acute preference and standard deviation of individual crayfish tested more than once. The final preferendum for the species is indicated in the last column, ±1 standard error

Species	Mean acute preference (°C)	SD	*N*	Final preferendum (°C)
P. clarkii	20.86	2.43	16	21.96 ± 0.65
	24.09	1.35	11	
	23.65	1.97	10	
	25.88	0.70	6	
	24.15	2.56	6	
	23.53	1.12	6	
P. spiculifer	21.89	1.78	11	23.4 ± 0.4
	20.18	2.12	6	
C. latimanus	20.15	1.73	11	16.8 ± 0.9
	20.06	1.05	6	

Table 2. The mode and mean of the acute preference for four individual *Procambarus spiculifer* determined during their first 2 hr in the gradient. The final preferendum for the same animals determined after 24–72 hr in the gradient

	Acute preference		
Sex	Mode (°C)	Mean (°C)	Final preferendum (°C)
Female	25	23.6	21
Male	21	23.0	24
Male	22	22.8	21
Male	22	24.2	24

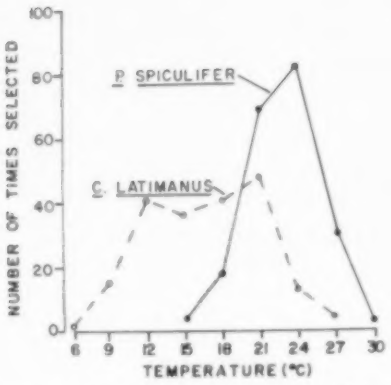

Fig. 2. The number of times that members of the species were observed within a 3°C temperature range, centering around the temperature illustrated. *Cambarus latimanus* is depicted by the dashed line and *Procambarus spiculifer* by the solid line. Observations were made within 150 min of placing a specimen in the gradient.

The acute preference of *Procambarus spiculifer* and *Cambarus latimanus* scatter about their final preferendum (Fig. 2). Preliminary studies had showed no acclimation effects, and neither species exhibited a significant difference between summer and winter determinations ($P > 0.05$). *C. latimanus* showed no change in diel preference, but *P. spiculifer* exhibited a circadian cycle that was minimum in the morning, reached a peak in the afternoon and started back down in the evening (Fig. 3). Morning and afternoon choices differed significantly ($P < 0.001$). A similar temporal pattern was observed in habitat temperature in both the second and the fifth-order streams. The magnitude of the habitat temperature change was directly related to weather conditions (Fig. 3), being greatest on a clear day, intermediate on a partly cloudy day and small to absent on cloudy or rainy days. The magnitude of the preference change was 3.5°C, closely matching the clear day AM to PM habitat temperature change of 3.43°C.

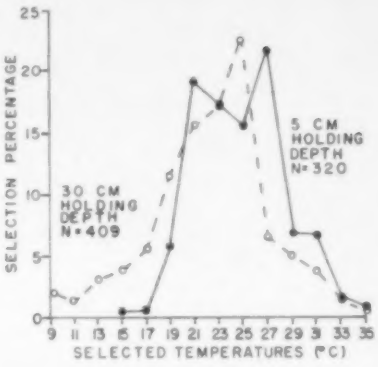

Fig. 4. The acute preference of *Procambarus clarkii* as a function of the water depth in their holding tanks.

The acute preference of *Procambarus clarkii* showed similar significant daily fluctuations (morning to afternoon, $P < 0.02$) as well as changes dependent on the water depth in their holding tank. The acute preference of animals held in 30 cm water and those held in 5 cm water differed significantly ($P < 0.0001$). When held in 30 cm water the mean acute preference was 23.26 ± 0.24°C and rose in 5 cm water to 25.36 ± 0.19. Not only was the mean shifted but the entire curve was likewise shifted (Fig. 4). Animals held in deeper water showed many more low temperature and fewer warm water choices. The plateau around 21°C corresponds to the final preferendum of *P. clarkii*.

The holding depth also produced some very obvious behavioral changes. In the shallow water holding

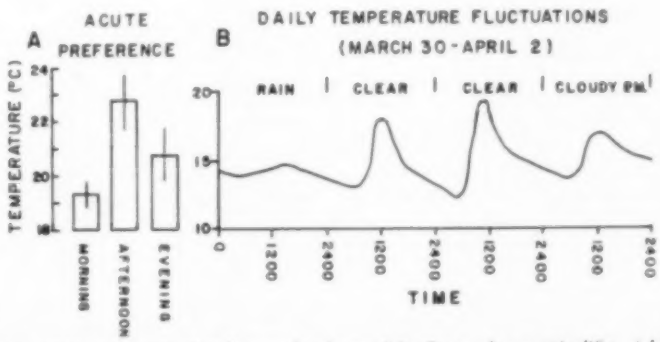

Fig. 3. (A) The acute preference circadian cycle observed in *Procambarus spiculifer*. ± 1 SE = bar. (B) Daily fluctuations for the days indicated for three different weather conditions. Continuous recordings were obtained with Ryan recording thermographs.

tanks they held the body flat on the bottom, legs spread and chelae extended anteriorly. They were not aggressive to conspecifics, rested in groups in the aquarium corners, appeared lethargic as if at rest, and had to be prodded to respond. In the deeper water group the body was carried high above the bottom with the claws extended laterally and above the level of the body. These animals exhibited many more aggressive encounters, maintained greater inter-individual distance, were more responsive to humans, and did appear to rest.

DISCUSSION

During June, the month of maximum activity for *Procambarus spiculifer*, temperatures increased from 23.5 to 27°C ($\bar{x} = 25.5$°C) in the fifth-order stream and from 19 to 22°C ($\bar{x} = 20.3$°C) in the second-order. The final preferendum of *P. spiculifer* was 23.4°C which closely corresponds to the temperatures experienced during the maximum activity period (open squares, Fig. 1). *Cambarus latimanus*, most active in October and November, also experienced water temperatures that matched their final preferendum. In the second-order stream the temperature stayed essentially constant at 15.7°C from 14 October to 28 November. *C. latimanus* was not found in the fifth-order stream but was numerous in the second-order stream during the time the temperature was constant and very close to their final preferendum of 16.8°C (solid square, Fig. 1). It would appear that for these two species, active for only a small part of the year, the final preferendum approximately equals the ambient temperature during their period of maximum activity.

Procambarus clarkii was used to test the above correlation because of its close systematic relationship of *P. spiculifer* and because its latitudinal distribution completely overlaps that of *P. spiculifer* (Hobbs, 1962, 1981; Rhoades, 1944). The two species are, consequently, exposed to very similar annual temperature regimes. *P. clarkii*, however, have their maximum period of activity earlier in the spring (Viosca, 1939; Penn, 1956; Huner, 1975), retreating into their burrows about the time that *P. spiculifer* becomes active. The final preferendum of *P. clarkii* of 21.96°C closely corresponds to the April and May habitat temperatures in Louisiana as measured by Penn (1943).

Thus, in all three species, the final preferendum matched the ambient temperature the species would experience during their peak activity periods. Although findings in fish relate their final preferendum to their zoogeographical distribution, this correlation does not appear to differ from the situation in the crayfish. In both groups the end result is the same, i.e. activity at temperatures close to the final preferendum.

The acute preference of both *Procambarus spiculifer* and *Cambarus latimanus* generally seem to cluster around their final preferendum *P. spiculifer* showed a sharper preference than did *C. latimanus* which probably could be best described as exhibiting no preference but avoiding both extreme cold and warm temperatures. *P. clarkii*, whose habitat is more lentic than the other species, showed an acute preference that was separated from its final preferendum, although a plateau was observed even in their acute preference that centered around their final preferendum (Fig. 4). Also, when *P. clarkii* was held in 5 cm water, they exhibited both an acute preference that was higher and a behavior that was less aggressive toward conspecifics than did those held in 30 cm water. These changes appear to be adaptations to a life history that is associated with changes in habitat water depth. In early summer, *P. clarkii* experience reduced water depths and increasing water temperatures, at which time they retire into their burrows. Often males and females occupy the same burrow (Payne, 1978). The reduced aggressiveness and other behavioral changes would seem to adapt them to cramped burrow conditions. The changes in acute preference fit the same pattern as the final preferenda discussed above, i.e. the preferred temperature (acute in this case) approximately equals the habitat temperature that the crayfish will experience. The same was true of the acute preference of *P. spiculifer* (see below).

Preference for the ambient temperatures that they will be exposed to over a daily or seasonal cycle initially appears illogical, until the habitat is considered. Turbulent flow in a stream prevents spatial variation in temperature (Williams, 1981); consequently, no behavioral thermoregulation is possible (Neill, 1979). Thermoregulation involves choosing from a range of spatially distributed temperatures and thus regulation of body temperature at the preferred temperature. Reynolds and Casterlin (1979) in fact equated temperature preference to behavioral thermoregulation. The preference data presented here are probably involved in a response to temporal changes in habitat temperature, a part of a behavioral arousal system. The difference between the preferred (T_p) and ambient (T_a) temperature or changes in the same two could be used as an error signal similar to that proposed by Neill (1979) to account for predictive thermoregulatory responses. Both *Cambarus latimanus* and *Procambarus spiculifer* hibernate. They appear at a species specific time in the spring, in a premolt condition (Taylor, unpublished) and heavily stained by red clay. They very seldom appear in the same condition at any other time of the year, and never in the numbers observed in the spring. The T_p–T_a difference could inform the crayfish, hibernating in a burrow, of the external conditions.

The acute preference of the two procambarid species could likewise indicate ambient conditions or time of day or night. A close correlation exists between light intensity or time of day and stream temperatures (Fig. 3), thus either can be used with equal precision as a cue for activity. The T_p–T_a difference in a retreat would indicate time of day and weather conditions as well. Support for the use of the T_p–T_a difference comes from both species. *C. latimanus*, who did not show the daily temperature preference cycle, is not activated by daily conditions but by the rising water levels following a heavy afternoon or evening rain (Taylor, 1983). *P. spiculifer* spends the daylight hours either in shallow burrows or under undercut banks (Hobbs, 1981). In the second-order stream the undercuts may be over 1 m;

the water is generally very turbid and after the riparian vegetation has leafed out the light intensity is fairly low. Under these conditions it is possible that the daily temperature cycle is a more accurate indicator of conditions outside the refugia than is light. I did not measure soil temperature in and around the stream, thus this postulate must remain tentative. Temperature serves as a *zeitgeber* for the circadian and circatidal rhythms of *Carcinus* (Williams and Naylor, 1969; Williams *et al.*, 1979) and has been implicated as a cue for seasonal and circatidal rhythms in an esturine amphipod (Holmström and Morgan, 1979).

I would conclude that the two species of stream crayfish utilize temporal thermal heterogeneities (spatial thermal heterogeneity is very rare) not for the purpose of thermal regulation but rather as a part of a daily and annual error detection system. The final preferendum of the swamp dwelling *Procambarus clarkii* closely matched their habitat temperature during their peak activity thus supporting the above conclusion.

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INFLUENCE OF PREGNANCY ON OXYGEN CONSUMPTION, HEART RATE AND HEMATOLOGY IN THE GARTER SNAKE: IMPLICATIONS FOR THE "COST OF REPRODUCTION" IN LIVE BEARING REPTILES

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Abstract—1. Oxygen consumption (V_{O_2}) was measured in pregnant (PR), postpartum (PP), nonpregnant (NP), neonatal and male garter snakes, *Thamnophis sirtalis*. Hematocrit, hemoglobin concentration and blood O_2 carrying capacity were measured for all groups except neonates, and heart rate was measured in PR and PP snakes.

2. Pregnancy significantly increased oxygen consumption, weight-specific V_{O_2}, and heart rate relative to PP individuals.

3. The magnitude of the increase in V_{O_2} in PR snakes was related to the number of fetuses.

4. No differences in the measured hematological parameters were observed between any group.

5. We propose that the V_{O_2} of PR snakes contains three major components: the females' resting V_{O_2}, fetal V_{O_2}, and the "cost of supporting the pregnancy". At term, fetal V_{O_2} and the cost of supporting the pregnancy are nearly equivalent and we suggest that in the evolution of live bearing reproductive habits physiological energetics plays an important role.

INTRODUCTION

Live bearing as a mode of reproduction occurs with surprising frequency throughout the animal kingdom (Hogarth, 1976). Despite the relative commonness of live bearing our knowledge of the maternal physiology associated with it outside the placental mammals is limited. This study was designed to examine some cardiorespiratory parameters of the garter snake (*Thamnophis sirtalis*) during late pregnancy.

Previous research on reptilian pregnancy is quite limited. The endocrinological literature was recently reviewed by Amoroso *et al.* (1977). In addition, Pough (1977) and Grigg and Harlow (1981) have described a difference in fetal and maternal blood oxygen affinities and Clark and Sisken (1956) investigated fetal nitrogen excretion. Studies specifically related to ours, however, are limited to two. Clausen (1936) and Guillette (1982) studied the oxygen consumption of pregnant *Stoeria dekayi* and *Sceloporus aeneus bicanthalis* respectively. Both investigators reported a significant elevation in weight-specific metabolism in pregnant vs nonpregnant or postpartum invididuals. However, because of small sample sizes, larger data scatter, and/or missing data, an analysis of the possible implications of these elevations in metabolism is difficult.

In this study, by obtaining more metabolic data on pregnant snakes just before parturition and other reproductive classes, in addition to information on heart rate and hematology, we hoped to be able to gain a greater insight into whether concomitant changes occur with the greater oxygen demand to facilitate oxygen transport. With this information, greater knowledge of the physiology of pregnancy in reptiles, how it compares with mammals, and the cost of live bearing as a mode of reproduction, might be gained.

MATERIALS AND METHODS

Pregnant, nonpregnant and male eastern garter snakes *Thamnophis s. sirtalis* were collected in northern Michigan and northwestern Ohio between April and June 1981 and 1982. Animals were maintained in either a large galvanized tank or aquaria at 25°C with a 12:12 day:night light cycle. Snakes were fed earth worms twice a week and water was available *ad libitum*. Animals were acclimated to laboratory conditions for a minimum of 4 weeks before measurements were made.

Oxygen consumption of adult male, nonpregnant female, postpartum female, and pregnant (within 1 week of parturition) snakes was measured at least 48 hr postprandially. Measurements were made between 10.00 and 15.00 hr at 25°C in a constant pressure volumetric apparatus with compensating chamber (McDonald, 1976). Soda lime was used as a carbon dioxide absorbant. Animals were placed in 473 ml metal containers, submerged in a water bath in 25°C constant temperature room, and allowed to acclimate at least 1 hr before oxygen consumption was recorded. Snakes remained isolated in the dark, totally undisturbed for the duration of each experiment. Oxygen consumption was measured until a steady state was achieved as indicated by a constant V_{O_2}. Oxygen consumption of pregnant animals was measured once a week until parturition. This was done in order to obtain a measure of oxygen consumption as close as possible to parturition and therefore maximum embryonic size (Clark *et al.*, 1955). Postpartum

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oxygen consumption measurements were made within 1 week of parturition. Because of their small size, neonatal snakes were studied in apparatus with 100 ml chambers.

In a separate group of snakes heart rate was measured with external adhesive ECG electrodes connected to the cardioscope of a Physio 1440 defibrillator. Electrodes were placed directly over the heart and just cephlad to the cloacal opening. To allow the snakes to acclimate to the electrodes they were attached the night before rates were measured. The snake was then isolated in a styrofoam box at 25°C until all measurements were completed. Measurements of heart rate were taken for 5 min approximately every hour between 9.00 and 18.00 hr and averaged. All recordings which indicated snake movement were not included in the final analysis.

Blood samples were obtained from ketamine anesthetized snakes. A mid-ventral incision was made caudal to the heart and approximately 1.0 cm^3 of blood was drawn from the dorsal aorta into a previously heparinized syringe. Hematocrit was determined by spinning blood for 5 min in a micro hematocrit centrifuge. Hemoglobin concentration was determined as cyan-methemoglobin. Blood oxygen carrying capacity was determined on blood equilibrated with 40% oxygen and 5% carbon dioxide at 25°C in an Astrup microtonometer for 10 min using the method of Roughton and Scholander (1943). Blood was not collected from neonatal snakes because of their small size.

Differences between means were determined using a Student's *t*-test. A *P* value of less than 0.05 was considered significant.

RESULTS

The oxygen consumption measurements are summarized in Table 1. Pregnant garter snakes have a significantly greater oxygen consumption and weight-specific oxygen consumption compared with postpartum females. Oxygen consumption of pregnant snakes was also significantly related to the number of fetuses the female was carrying (Fig. 3). No significant differences were observed between postpartum and nonpregnant female snakes.

To examine variations in metabolic rate not primarily connected to body weight the metabolic rate per metabolic unit of body weight ($g^{0.76}$) was calculated (Table 1). It appears that differences in body mass account for the differences in V_{O_2} between males, nonpregnant females, and neonates, i.e. there is no evidence for an ontogenetic or influence due to sex in our V_{O_2} data.

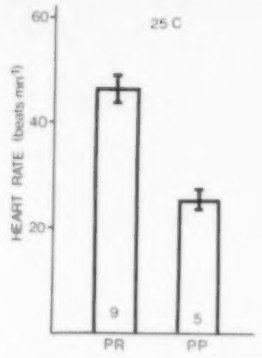

Fig. 1. Heart rates of nine pregnant (PR) and five postpartum (PP) garter snakes. Difference is statistically significant at the 0.001 probability level.

Heart rate measurements are shown in Fig. 1. The mean heart rate of pregnant snakes was significantly higher than that of postpartum snakes ($P < 0.001$). Increases ranging from 40 to 130% were observed in individual snakes between the pregnant and postpartum condition.

Hematological results are shown in Table 2. Neither pregnancy nor sex appears to influence the parameters we measured.

DISCUSSION

Hematology

Pregnancy does not appear to affect the blood parameters we measured (Table 2). This observation is in contrast to the changes observed in some pregnant mammals where hemodilution may occur (Hoversland, 1974a), but consistent with our own studies on another snake *Agkistrodon piscivorus* where no effects were observed (Birchard *et al.*, 1984). The values obtained here for hematocrit, hemoglobin concentration, and blood oxygen carrying capacity are very similar to those reported by Pough (1977) for this same species.

Heart rate

We are not aware of data on the effect of pregnancy on heart rate in nonmammalian vertebrates.

Table 1. Body weights and oxygen consumption of *Thamnophis sirtalis* ($\bar{X} \pm$ SE (N))

	Body weight (g)	Oxygen consumption (cm^3O_2 STP·hr^{-1})	(cm^3O_2 STP·hr^{-1}·g^{-1})	(cm^3O_2 STP·hr^{-1}·$g^{-0.76}$)
Males	30.3 ± 2.0 (6)	1.75 ± 0.24 (6)	0.059 ± 0.001 (6)	0.133 ± 0.02 (6)
Nonpregnant females	52.1 ± 4.4 (3)	2.89 ± 0.84 (3)	0.054 ± 0.011 (3)	0.140 ± 0.03 (3)
Postpartum females	52.5 ± 1.8 (4)	3.26 ± 0.05 (4)	0.065 ± 0.005 (4)	0.167 ± 0.12 (4)
Pregnant females	94.2 ± 10.2 (6)*	†8.60 ± 1.16 (7)	†0.095 ± 0.010 (6)	†0.282 ± 0.03 (6)
Neonates	1.66 ± 0.07 (8)	0.20 ± 0.01 (8)	0.119 ± 0.010 (8)	0.134 ± 0.01 (8)

*One individual's weight was not recorded.
†Significantly different from postpartum females $P < 0.001$.

Table 2. *Thamnophis sirtalis* hematology ($\bar{X} \pm$ SE)

		Hematocrit (vol. %)	Hemoglobin (g %)	O_2 capacity (vol. %)
Males	8	30.4 ± 1.63	9.12 ± 0.05	9.6 ± 0.7
Nonpregnant females	3	32.6 ± 3.5	10.10 ± 1.20	9.2 ± 1.2
Pregnant females	7	29.3 ± 2.0	9.40 ± 0.50	11.1 ± 0.6
Postpartum females	6	33.3 ± 1.5	10.00 ± 0.50	9.7 ± 1.0

However, the large increase observed here is similar to the pattern seen in mammals (Hoversland *et al.*, 1974a; Hytten and Leitch, 1971). The combination of our observations of an increased V_{O_2} and an unchanged blood oxygen carrying capacity would suggest that cardiac output increases during pregnancy to supply fetal oxygen demands.

Oxygen consumption

Pregnancy in garter snakes results in significantly higher oxygen consumption, $V_{O_2} \cdot g^{-0.76}$, and weight-specific oxygen consumption rates late in gestation (Table 1). Clausen (1936) and Guillette (1982) also observed an increase in weight-specific oxygen consumption in *Storeria dekayi* and *Sceloporus aeneus bicanthalis* respectively. That pregnant snakes exhibit a greater oxygen consumption is to be expected on the basis of their greater body weight (Table 1). However, the fact that the weight-specific oxygen consumption rates of pregnant snakes are greater than those of postpartum snakes is contrary to allometric predictions based on body weight (Bennett and Dawson, 1976). We believe the increased $V_{O_2} \cdot g^{-0.76}$ and weight-specific oxygen consumption indicates that fetal oxygen consumption is scaled to fetal and not maternal weight.

That fetal metabolism at term in reptilian live bearers is scaled to fetal weight is consistent with similar observations in oviparous reptiles (Seymour, 1979). In birds Hoyt and Rahn (1980) have shown that embryonic metabolism appears to be scaled to maternal body mass but after hatching changes rapidly to be proportional to neonatal mass. This same pattern was suggested for mammals by Kleiber (1965) but more recent investigations indicate the metabolic pattern of pregnant vs postpartum female mammals is similar to what has been observed here for reptiles and that fetal and neonatal oxygen consumptions are also quite similar (Clapp *et al.*, 1971; Hoversland *et al.*, 1974a; Parer, 1978). The major differences between fetal and neonatal oxygen consumptions proposed for mammals in the past appear to have been the result of the techniques used (see Clapp, 1971 for review), improper scaling of neonatal V_{O_2} (Poczopko, 1979) and not the result of maternal control over fetal metabolism as proposed by Kleiber (1965).

To examine further the significance of the total increase in oxygen consumption observed in pregnant garter snakes an analysis like that illustrated in Fig. 2 was conducted. In four snakes pregnant and postpartum oxygen consumption data were available as well as the total number of live neonates produced. In this analysis it was assumed that neonatal and fetal oxygen consumptions were equal (oxygen consumption of the fetal membranes was neglected because of their small mass). Thus, for each pregnant snake a predicted total fetal V_{O_2} was calculated as neonatal V_{O_2} times the number of neonates. As can be seen in Fig. 2, for one representative female the sum of the postpartum and predicted fetal oxygen consumptions does not equal that of the pregnant snake by an amount labeled Δ. This was true for all four snakes examined. We feel the magnitude of this value is too large to be entirely accounted for by the metabolism of the fetal membranes. Therefore this analysis indicates that oxygen consumption of a

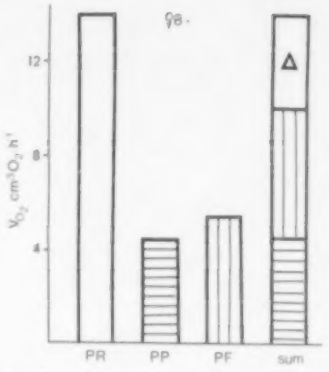

Fig. 2. Partition of oxygen consumption in one representative garter snake. Pregnant (PR), postpartum (PP), and predicted fetal (PF) oxygen consumptions are shown. See text for explanation.

pregnant snake is comprised of three major components: the standard metabolism of the female, the total fetal oxygen consumption, and Δ, the "cost of supporting the pregnancy".

There is a significant relationship ($P < 0.05$) between the Δ values of the four pregnant snakes and the number of live neonates at birth (Fig. 3). The slope indicates the increased cost per neonate at term that a female snake must invest to support that fetus (in this analysis $0.17 + 0.03$ $cm^3O_2 \cdot hr^{-1}$). In other words at term the metabolic investment by the mother is nearly equivalent to that of neonatal (fetal) V_{O_2}. We assume that this cost is the result of the many maternal processes associated with supporting developing fetuses. For example, supplying oxygen to the fetuses (the increased heart rate observed here) and the maternal handling of fetal nitrogenous wastes (fetuses of live bearing reptiles produce ammonia and urea (Clark and Sisken, 1956)) which are transfered to the maternal circulation and excreted in part as uric acid.

The intercept of the regression shown in Fig. 3 indicates whether or not any basic changes have occurred in maternal metabolic rate during pregnancy. A negative value like the one shown here would be indicative of a lowering of this value, and

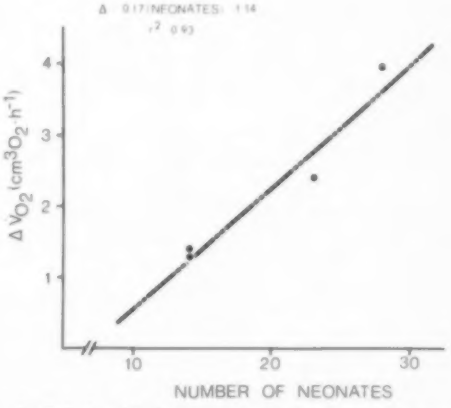

Fig. 3. Relationship between "cost of supporting the pregnancy" and the number of neonates.

if real, would potentially represent a great advantage to the female snake in that energy could be conserved at a time when fuels to support fetal development are needed. In our analysis we cannot draw any definite conclusions about the intercept for two reasons. Firstly, because of the limited number of points available the confidence interval is large and spans into the positive range, and secondly the intercept is dependent on the assumption we have made about the level of fetal metabolism.

The "cost of supporting the pregnancy" as observed here is of physiological interest and ecological and evolutionary import. Unfortunately we are aware of only one other analysis similar to our own. Hytten and Leitch (1971) using calculated $\dot{V}_{O_2}$ values for various tissues concluded the additional oxygen consumption observed during pregnancy in humans was due to a combination of fetal and fetal membrane oxygen consumption and the increased ventilatory and cardiac work done during pregnancy. This comparison, although consistent with our reptilian results, is limited given the small proportion of the total body weight the uterine contents represent in man and thus the small increase in $\dot{V}_{O_2}$ that would result.

Oxygen consumption in postpartum female snakes was somewhat but not significantly elevated above that of nonpregnant females (Table 1). Whether this is indicative of some residual change in female standard metabolism during pregnancy, perhaps caused by a hormonal difference like the cardiorespiratory changes caused by progesterone in mammals (see Hoversland *et al.*, 1974a,b for review), or is artifactual, will require further study.

Ecological and evolutionary implications

The "cost of reproduction", particularly in reptiles, has commonly been inferred from the weight and/or caloric contents of either the gonads or eggs of a female even with the acknowledged problems of this measure (Congdon *et al.*, 1982; Tinkel and Hadley, 1975). Although we have only very limited data on oviparous species for comparison (Guillette, 1982) the present study indicates that in a live bearing animal this methodology would result in a significant underestimation of the maternal energy that has been invested per young. Further, our data suggest that energy which might potentially be invested in eggs and thus more offspring and/or growth may have to be saved, or obtained during pregnancy (exposing the female to greater predation), to support the developing fetuses. Analysis of the data of Tinkle and Hadley (1975) shows that the body energy content of the viviparous lizard *Sceloporus jarrovi* is greater relative to the 13 other species studied, which were all oviparous. The greater body energy content provides some indirect evidence for the strategy of energy stores being built up before pregnancy. The results presented here also indicate that the calculation of the total energy budget for *S. jarrovi* presented by Tinkle and Hadley (Table 7, 1975) is an underestimation, as are any calculations of reproductive effort in live bearing reptiles which do not include what we have described as the "cost of supporting the pregnancy". It appears that live bearing is a physiologically much more expensive mode of reproduction than might have been indicated by previous investigations. Because of the importance of the way calories are partitioned and used by various physiological compartments (maintenance, growth and reproduction) in influencing life history strategies (Calow, 1979; Hirshfield and Tinkle, 1975) we believe the factors selecting for the evolution of live bearing to have been substantial.

Acknowledgements—Funds to support this research were provided by the Faculty Research and Development Fund of the University of Toledo Graduate School awarded to CPB. GFB was supported in part by NHLBI Training Grant No. HL07449-02. We wish to thank K. Schuett and A. Rio for help in collecting snakes. D. L. Kilgore, Jr, J. R. Stewart and D. Bartlett made helpful comments on earlier drafts of this manuscript.

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EFFECT OF TEMPERATURE AND INHIBITORS ON SPORULATION AND THE CALCIUM ACCUMULATION SYSTEM IN *BACILLUS MEGATERIUM*

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Abstract—1. Incubation at 37°C seems to prevent the formation of the calcium accumulation system during sporulation in *Bacillus megaterium*.

2. The temperature sensitive period for the formation of the calcium accumulation system was approximately at the end of growth.

3. KCN inhibited not only the formation of the calcium accumulation system but also calcium uptake, whereas chloramphenicol inhibited the formation of the calcium accumulation system alone.

INTRODUCTION

Most bacterial cells keep the intracellular calcium concentration lower than the extracellular calcium, as a consequence of the extrusion of calcium by active transport (Silver and Kralovic, 1969; Silver *et al.*, 1975; Devés and Brodie, 1981). The everted vesicles of the bacterial cells can accumulate calcium. Calcium accumulation has been shown in the everted vesicles of *Escherichia coli* cells (Rosen and McClees, 1974; Tsuchiya and Rosen, 1975, 1976), and in the vesicles of other bacterial cells (Golub and Bronner, 1974; Bhattacharyya and Barnes, 1976; Kobayashi *et al.*, 1978; Belliveau and Lanyi, 1978; Barnes *et al.*, 1978; Kumar *et al.*, 1979). Calcium accumulation in *Bacilli* is known to occur during sporulation (Young and Fitz-James, 1962; Pearce and Fitz-James, 1971; Bronner *et al.*, 1971; Bronner and Freund, 1972; Hogarth and Ellar, 1978). An active transport system for calcium existed in sporulating *Bacillus megaterium* (Hogarth and Ellar, 1979) and *B. subtilis* (Eisenstadt and Silver, 1972). Vesicles from sporulating cells of *B. megaterium* were supposed not to accumulate calcium. However, they could take up calcium as the vesicles of vegetative cells of this bacterium (Golub and Bronner, 1974) and those of other bacterial cells. This observation was explained by the assumption that both calcium uptake and efflux systems coexist in the sporulating cells of *B. megaterium* (Ota, 1980a). The assumption was supported by the fact that there was an exchange of calcium accumulation in the cells for external calcium in the medium (Ota, 1982a).

Although *B. megaterium* mutant 3-12 cannot sporulate owing to a lack of dipicolinic acid synthesis, calcium uptake occurred during the period which corresponded to the calcium uptake by the wild-type strain in the sporulation sequence and the ability of calcium uptake decreased after attaining its maximum (Ota, 1980a, 1982a). The mutant could sporulate on the addition of dipicolinate (Fukuda and Gilvarg, 1968), while dipicolinic acid-less mutants of *B. cereus* were reported to form heat sensitive spores in the absence of added dipicolinate (Wise *et al.*, 1967). A mutant capable of sporulation but unable to accumulate calcium could not be isolated, and the amount of the calcium accumulation correlated with the sporulation efficiency in the mutant when dipicolinate was added (Ota, 1980a). Heat-sensitive spores were formed in the absence of calcium in the wild-type strain of *B. megaterium* (Tamir and Gilvarg, 1966). From these results, it was suggested that calcium accumulation in the cells was not essential for sporulation but that the change in the cells which evoked calcium uptake was essential (Ota, 1980a). Several works have shown the property of calcium uptake during sporulation (Hogarth and Ellar, 1978, 1979; Eisenstadt and Silver, 1972; Ota, 1982a; Seto-Young and Ellar, 1981).

This paper describes the influence of temperature and inhibitors on the formation of the calcium accumulation system and some properties of calcium accumulation in the process of sporulation in the wild-type strain of *B. megaterium* and its asporogenous mutant. In addition, other factors which affect sporulation and morphogenesis in bacteria are discussed.

MATERIALS AND METHODS

Organisms

A local strain (wild-type) and a mutant 3-12 of *B. megaterium* (Fukuda and Gilvarg, 1968) were used. The mutant 3-12 lacks dihydropicolinate synthase (EC 4.4.1.52) and needs diaminopimelate and lysine for growth. They were isolated from *B. megaterium* local strain after the treatment of *N*-methyl-*N'*-nitro-*N*-nitirosoguanidine (Fukuda and Gilvarg, 1968; Adelberg *et al.*, 1965).

Media

The minimal medium (FCG) (Millett and Aubert, 1960), which was used for growth, excluded yeast extracts for the local strain, and then included diaminopimelic acid (0.005%) and L-lysine–HCl (0.01%) for the mutant strain (Fukuda and Gilvarg, 1968).

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Growth and sporulation condition

Cells were grown on a rotary shaker containing 25 ml at 32°C with vigorous aeration and at the end of growth, 2.5 μmol of calcium labelled with ^{45}Ca (2.5×10^3 counts/min nmol^{-1}) was added. In the measurement of calcium accumulation during 30 min, calcium was not added to the culture at the end of the growth. The vegetative growth of cells ceased on reaching 120–130 Klett units at 660 nm in the turbidity, and pH was maintained at 6.5–6.8 throughout growth and sporulation. The temperature in the culture was shifted from 32 to 37°C in the experiment on the influence of temperature.

Purity of mutant 3-12

The purity of mutant 3-12 was examined by seeing whether or not the organisms could grow on a plate in the absence of mesodiaminopimelate and L-lysine–HCl. The wild-type strain which was diluted 1000-fold with F media minus NH_4Cl (pH 6.8) was grown on the other plate as a control. Contamination of the mutant 3-12 with the wild-type was estimated at less than 10^{-6}.

Measurement of calcium accumulation

Each 0.1 ml of the medium was taken from the culture flask at a proper time and put into 5 ml of the solution (the culture medium minus glucose and $CaCl_2$). The mixture was shaken vigorously and placed in duplicate on membrane filters, which were washed thoroughly with 30 ml of the solution described above, and counted for radioactivity with a Packard 3003 Tri-Carb liquid scintillation counter. The scintillation fluid was prepared by Bray's (1960) method.

Measurement of calcium accumulation during 30 min

One millilitre of cells cultured without added calcium was taken from the culture flask at a proper time and put into a tube containing 100 nmol of the calcium labelled with ^{45}Ca (2.5×10^3 counts/min nmol^{-1}). Next after 30 min at 32°C with vigorous aeration, 0.1 ml was taken from the tube and the following procedure was the same as described in the measurement of calcium accumulation.

Measurement of sporulation efficiency

The colony forming units of the sporulating culture determined after heat treatment for 15 min at 60°C were taken as a spore titre. The sporulation efficiency was also measured directly by counting cells containing spores in a Petroff Hauser chamber with the oil immersion lens of a phase contrast microscope. The specific pattern of the decline and rise of the culture turbidity during sporulation was used as a measure of sporulation efficiency (Fukuda and Gilvarg, 1968).

RESULTS

Influence of temperature on calcium accumulation and sporulation in wild type

Influence of higher temperature on calcium accumulation during sporulation process in wild-type strain of *B. megaterium* is shown in Fig. 1. The wild-type strain of *B. megaterium* incubated at 32°C exhibited a secondary rise of its turbidity curve (Fig. 1). The specific pattern of this decline and rise of culture turbidity was conveniently used as a measure of sporulation efficiency (Fukuda and Gilvarg, 1968). A large amount of calcium was taken up, corresponding to the secondary rise, in the cells which had been incubated at 32°C as previously indicated (Ota, 1980a). Cells incubated at 37°C reached their end of growth about 2.5 hr earlier than cells incubated at

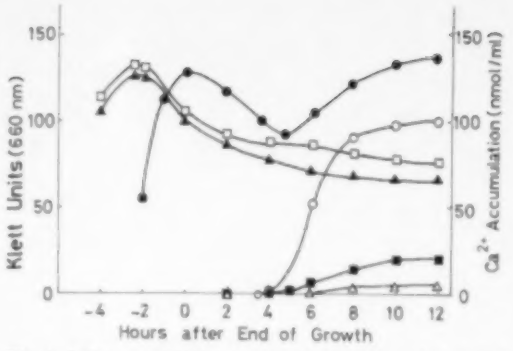

Fig. 1. Influence of higher temperature on sporulation and calcium accumulation in wild-type strain. Wild-type cells were grown at 32°C or 37°C with vigorous aeration. The calcium labelled with ^{45}Ca were added to their culture at the end of growth. A 0.1 ml aliquot of the culture was taken at the indicated time and the calcium accumulation was measured. Detailed conditions and the procedure of the measurement are described in the Materials and Methods section. ●, Turbidity curve of culture in the wild-type at 32°C; ▲, turbidity curve of culture in the wild-type at 37°C; □, turbidity curve of culture in the wild-type cultured in the medium containing 1 mM sodium dipicolinate at 37°C; ○, calcium accumulation in the wild-type cultured at 32°C; △, calcium accumulation in the wild-type cultured at 37°C; and ■, calcium accumulation in the wild-type cultured in the medium containing 1 mM sodium dipicolinate at 37°C.

32°C. After the end of growth, their turbidity curve declined throughout the time course. Calcium uptake occurred 6 hr after the end of growth, which was delayed about 2 hr, and its amount was very small. These phenomena seem to be related to the result that sporulation did not occur during incubation at 37°C.

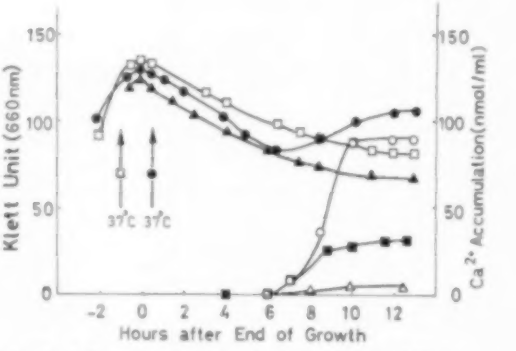

Fig. 2. Influence of timing in temperature shift up on sporulation and calcium accumulation in wild-type strain. Cells of the wild-type were grown at 32°C with vigorous aeration. At the indicated time, temperature was shifted from 32°C to 37°C following the same procedure as shown in Fig. 1. ▲, Turbidity curve of culture in the wild-type at 37°C; □, turbidity curve of culture temperature-shifted from 32°C to 37°C 1 hr before the end of growth in the wild-type; ●, turbidity curve of culture temperature-shifted from 32°C to 37°C at 0.5 hr after the end of growth; △, calcium accumulation by the wild-type culture at 37°C; ■, calcium accumulation by the wild-type temperature-shifted from 32°C to 37°C 1 hr before the end of growth; ○, calcium accumulation by the wild-type temperature-shifted from 32°C to 37°C 0.5 hr after the end of growth.

It was reported that temperature was strictly controlled because it was a critical factor in sporulation of the wild-type strain of *B. megaterium* (Fukuda and Gilvarg, 1968). Insufficient aeration during incubation lowered sporulation efficiency. An amount of calcium uptake was also decreased by the insufficient aeration during incubation. The calcium accumulation in the cells of the wild-type strain which had been incubated at 37°C increased to some degree in the presence of dipicolinate, but sporulation could not be observed. The amount of added dipicolinate allowed asporgenous mutant 3-12 to sporulate (Ota, 1980a; Fukuda and Gilvarg, 1968). The calcium accumulation of wild-type strain was different from that of mutant 3-12, for the former kept its level even after attaining its peak (Figs 1 and 2), whereas the latter decreased after its peak (Figs 3 and 4, Ota, 1980a).

Influence of timing in temperature shift on sporulation and calcium accumulation in wild-type

Influence of timing in temperature shift (32–37°C) on sporulation and calcium accumulation are shown in Fig. 2. When the temperature was raised to 37°C 1 hr before the end of growth, about 30% of the amount of calcium taken up at 32°C was observed. When the temperature was raised to 37°C 0.5 hr after end of growth, about 90% of the amount of calcium taken up at 32°C, and some sporulation, was observed about 2 hr later than the control.

Effect of inhibitors on calcium accumulation in mutant 3-12

Figure 3 indicates the effect of KCN on the formation of calcium accumulation system and on the decrease of calcium accumulation after attaining its peak in *B. megaterium* mutant 3-12. The calcium uptake was not observed when KCN was added to the culture which had been incubated at 32°C 2 hr after end of growth. The slope of the decrease curve of calcium accumulation becomes steeper on the addition of KCN. Since the calcium accumulation system contains both uptake and release systems (Ota, 1980a), the curve represents the amount of calcium remaining plus calcium taken up minus calcium released. A sharp decline of the curve after the addition of KCN is due to its inhibition of calcium uptake (Table 1). From this curve, it can be concluded that KCN does not inhibit the calcium release system.

Effect of chloramphenicol on calcium accumulation system is shown in Fig. 4. The formation of calcium accumulation system was inhibited completely by the addition of chloramphenicol 2 hr after end of growth. The decrease curve of calcium accumulation became a little steeper on the addition of chloramphenicol. Effect of KCN and chloramphenicol on the calcium accumulation limited to a 30 min period in *B. megaterium* mutant 3-12 is shown in Table 1. KCN at a concentration of 10^{-3} M and of 10^{-4} M inhibited 87% and 73% of the calcium accumulation of the control, respectively. About half the inhibition of the calcium accumulation in the control was shown by 10^{-5} M KCN. Chloramphenicol did not inhibit the calcium accumulation. Kumar *et al.* (1979) reported that KCN inhibited the respiration driven calcium uptake by the membrane vesicles of *Mycobacterium phlei*, while it had no effect on ATP-driven calcium uptake.

Influence of freezing on the formation of calcium accumulation system in mutant 3-12

Figure 5 shows the influence of freezing on the formation of calcium accumulation system in *B. megaterium* mutant 3-12. The culture was taken at

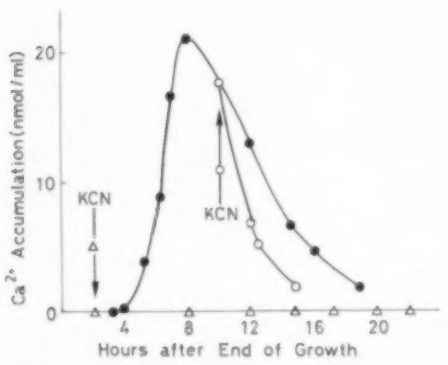

Fig. 3. Effect of KCN on calcium accumulation in mutant 3-12. Cells of mutant 3-12 were cultivated at 32°C with vigorous aeration and 2.5 μmol calcium labelled with ^{45}Ca were added at the end of growth. At the indicated time (arrows), 1 ml KCN was added to the mutant 3-12 culture. A 0.1 ml aliquot of the culture was taken at the indicated time and then the calcium accumulation was measured as described in the Materials and Methods section. ●, Accumulation of calcium in mutant 3-12; △, amount of calcium in mutant 3-12 after 1 mM KCN was added at 2 hr before the end of growth; ○, amount of calcium in mutant 3-12 after 1 mM KCN was added at 10 hr after the end of growth.

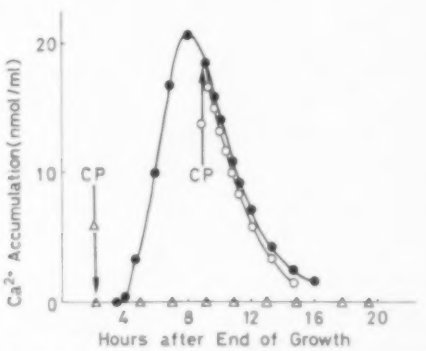

Fig. 4. Effect of chloramphenicol on calcium accumulation in mutant 3-12. Cells of mutant 3-12 were grown at 32°C with vigorous aeration, and 2.5 μmol calcium labelled with ^{45}Ca were added at the end of growth. At the indicated time (arrows) chloramphenicol (50 μg/ml) was added to the mutant 3-12 culture. A 0.1 ml aliquot of the culture was taken, and the calcium accumulation was measured by the procedure described in the Materials and Methods section. ●, Accumulation of calcium in mutant 3-12; △, amount of calcium in mutant 3-12 after chloramphenicol 50 μg/ml was added at 2 hr before end of growth; and ○, amount of calcium in mutant 3-12 after chloramphenicol 50 μg/ml was added at 9 hr after the end of growth.

Table 1. Effect of KCN and chloramphenicol on calcium accumulation in mutant 3-12

Inhibitors	Concentration	Calcium accumulation (nmol/ml)	Inhibition (%)
None		19.5	0
KCN	10^{-3} M	2.5	87
	10^{-4} M	5.3	73
	10^{-5} M	9.9	49
None		19.4	0
Chloramphenicol	50 μg/ml	19.8	0

Cells of mutant 3-12 were grown at 32°C with vigorous aeration. Calcium was not added to the culture at the end of growth. A 1 ml aliquot of the culture was taken from the culture flask when calcium accumulation would attain to its peak and put into a tube containing 100 nmol of calcium labelled with ^{45}Ca plus KCN or chloramphenicol. After 30 min at 32°C with vigorous aeration, 0.1 ml was taken from the tube and the following procedure was the same as described in the Materials and Methods section.

6 hr after the end of growth, added to glycerol in order to make a 10% glycerol solution and stored at −15°C for 64 hr. The stored cells were washed with medium to get rid of glycerol. The amount of calcium accumulation decreased on account of freezing, thawing and cell lysis due to the freeze–thaw, but maintained its original stage and then the same process proceeded in spite of the very low level of calcium accumulation. This indicates that the structure in the cells for calcium uptake system is not very labile to freezing and thawing under these conditions.

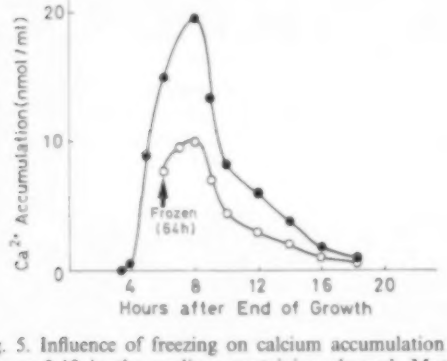

Fig. 5. Influence of freezing on calcium accumulation by mutant 3-12 in the medium containing glycerol. Mutant 3-12 cells were grown at 32°C with vigorous aeration. A 1 ml aliquot of the culture was taken from the culture flask at the proper time after end of growth, and the amount of calcium accumulated during 30 min was determined by the method of "Measurement of calcium accumulation during 30 min" as described in the Materials and Methods section. At the indicated time (arrows), 10 ml of the culture was taken and after the addition of glycerol in order to make a 10% solution, was stored at −15°C in a freezer. After 64 hr the stored culture cells collected by centrifuge were washed with the medium omitting glucose, yeast extract and calcium from medium FCG and supplementing with diaminopimelic acid (0.005%) and L-lysine–HCl (0.01%) in order to remove glycerol, then suspended in an original volume of the medium. The incubation was performed at 32°C. The accumulation of calcium was determined by the method of "Measurement of calcium accumulation during 30 min" as described in the Materials and Methods section. ●, Calcium accumulation in mutant 3-12 cells; and ○, calcium accumulation in mutant 3-12 cells taken at 6 hr after the end of growth and stored for 64 hr in a freezer.

DISCUSSION

Wild-type strains of *B. megaterium* could not sporulate at 37°C, and the amount of their calcium uptake observed in the sporulation sequence was very small (Fig. 1). However, when the temperature was raised up to 37°C 30 min after the end of growth, some sporulation and a considerable amount of calcium uptake were observed, although their period was delayed (Fig. 2). This result supports the previous conception (Ota, 1980a) that the change evoking calcium uptake in the cells is essential for sporulation in *B. megaterium*. The time required to reach the end of growth in the cells incubated at 37°C was shorter than that of the cells incubated at 32°C, whereas calcium uptake at 37°C started later than that at 32°C, and the amount was very small (Figs 1 and 2). This suggests that the temperature-sensitive change near the end of growth affects the structural changes for the formation of the calcium accumulation system which corresponds to a rise of the secondary turbidity curve later.

It is considered that dimorphism of fungal species is a good example of primitive morphogenesis (Haidle and Storck, 1966). *Blastomyces dermatilidis* can induce two morphological forms, a mycelial form and a yeast-like form. The organism grows as the mycelial form at 33°C and grows as the yeast-like form at 37°C (Levine and Ordal, 1946). The two morphogenetic patterns, M (mycelial) ⇆ Y (yeast-like) interconversion in *Blastomyces dermatilidis*, were called "thermal dimorphism" because that interconversion had been concluded to be solely a function of temperature (Nickerson, 1948).

Structural changes in the cells of *B. megaterium* for the formation of the calcium accumulation system during sporulation seem to resemble this phenomenon in the influence of temperature. Some of fungi for the M ⇆ Y interconversion are influenced by —SH compound, glucose, citrate, amino acids, metals, CO_2, O_2, auxin, age, pH, X-ray irradiation etc. other than temperature (Haidle and Storck, 1966; Nickerson, 1948; Pine and Peacock, 1958; Scherr and Weaver, 1953; Bartnicki-Garcia and McMurrough, 1971; Takada *et al.*, 1963; Johnson, 1954; Steele and Miller, 1974). Some bacteria can be induced to elongate into filaments by various treatments which are denoted B → F conversion (Nickerson, 1948).

Even in the case of thermal dimorphism, oxygen consumption increased in the high temperature range (Nickerson and Edwards, 1949). In this case, not only the oxygen consumption but also the content of cytochrome oxidase and related enzyme etc. possibly increased. Thus, temperature alone is not considered to be a factor in this case. Oxygen consumption is important for the conversion (Nickerson, 1948). It was observed that oxygen consumption increased after transfer to sporulation medium (Croes, 1967; Hopper *et al.*, 1974) and that the enzyme activities which may be related to the increase varied in yeast *Saccharomyces cerevisiae* (Ota, 1979, 1982b). In addition, some enzymes (Ota, 1980b, 1982c) and the transport activity (Ota, 1982d, 1983a,b), which might be related indirectly to oxygen consumption, increased. The spore formation was blocked by ethanol whose sensitive period was between 40 and 90 min after the end of growth in *B. subtilis*, which was considered to be the result of a physical change upon the cell membrane (Bohin *et al.*, 1976a). Spo0 mutant of *B. subtilis* had higher membrane-bound nitrate reductase activities than their Spo$^+$ parental strains. It was suggested that the increased nitrate reductase activity resulted from a mutational change in some membrane component other than the enzyme itself, that is, nitrate reductase in the modified membrane exhibited allotopic properties (Bohin *et al.*, 1976b). The term allotopy was first described by Racker (1967). Lundgren and Beskid (1960) isolated temperature-sensitive mutants of *B. cereus* ATCC 4342 in the minimal medium, and the sporulation interference was partially reversed when casein hydrolysate was added. Lundgren and Cooney (1962) reported that a temperature-sensitive asporogenous mutant of *B. cereus* took up about 30% as much of calcium as the parent during sporulation at 37°C.

Calcium uptake increased somewhat when added to dipicolinate and cultured at 37°C (Fig. 1). This increase is probably because that calcium is transported as calcium dipicolinate. Furthermore, it might be possible that calcium dipicolinate has an influence on the cell structure leading to increase in calcium uptake.

As the substances influencing M ⇆ Y conversion are various in different cultural conditions of fungi, fungous species etc., substances influencing sporulation are various in those of bacteria. A dipicolinic acid-less mutant of *B. cereus* is able to form heat-sensitive spores in the absence of dipicolinate (Wise *et al.*, 1967), but *B. megaterium* mutant 3-12 needs dipicolinate for its sporulation (Fukuda and Gilvarg, 1968). This indicates that dipicolinate participates not only in heat resistance of spores but also in the formation of spores in the case of the mutant 3-12.

Temperature-sensitive mutants of *B. subtilis* could sporulate at 47°C by the infection of a spore-converting bacteriophage PMB12, and the mutation to Spo (Ts) was suggested to be due to the mutation in either the RNA polymerase subunit or the 30 S ribosomal subunit. It was also suggested that PMB12 might have at least three genes for spore conversion and that the products interact with a host cell pathway which is expressed in the earliest stage of sporulation (Kinney and Bramucci, 1981). Young (1976) characterized two temperature-sensitive sporulation mutants of *B. subtilis*; one was obstructed at stage II and the cells exhibited morphological abnormalities, and the other was obstructed at stage IV to V when formation of the spore cortex was almost complete but coat formation was arrested. The former had a short temperature sensitive period during the formation of spore septum, and the product of the defective gene was suggested to be a protein. The m-RNA which coded for this protein was short-lived. It was suggested that coat formation of the latter was arrested at an early stage and that synthesis of the product of the defective gene started long before it assumed its physiological function. Both of them failed to synthesize dipicolinic acid. A temperature-sensitive DNA synthesis-mutant of *B. subtilis* has been isolated in which the process of initiation of DNA replication is inhibited at high temperature (Laurent and Vanier, 1973).

Haidle and Storck (1966) showed that RNA precursors were discontinuously incorporated into RNA during Y → M conversion in *Mucor rouxii*. It might be possible that in wild type of *B. megaterium* the temperature shift up affected one or more of these RNA polymerase of ribosome, the pattern of RNA synthesis, the DNA replication, the other enzymes or protein in the earliest stage of sporulation. Chloramphenicol inhibits the formation of the calcium accumulation system, but after the formation of the system it does not inhibit the calcium accumulation and the decrease of accumulation in the cells (Fig. 4 and Table 1).

Chloramphenicol prevents protein breakdown as well as sporulation, but once the protein degradation is underway it does not diminish its rate, although protein synthesis and sporulation are completely blocked (Kornberg *et al.*, 1968). KCN inhibited both calcium uptake and the formation of the calcium uptake system (Table 1 and Fig. 3). This supports the previous suggestion that most of the calcium uptake is energy-dependent both in the mutant 3-12 (Ota, 1982a) and the cells of sporulating *Bacilli* (Hogarth and Ellar, 1979; Eisenstadt and Silver, 1972). Bronner *et al.* (1975) reported that energy-dependent calcium exclusion system was preserved until T_6 stage in sporulation in *B. megaterium*. It is not likely that KCN inhibits the decrease of the accumulated calcium (Fig. 3). This means that a large part of the calcium release of asporogenous cells in this stage is energy independent, and KCN does not inhibit the decrease or degradation of calcium accumulation system.

I propose a tentative model of cell differentiation as follows:

Vegetative cell / Undifferentiated cell → (I factors: change of gene expression etc.) → (M factors: change of membrane components and membrane structure etc.) → Spore / Differentiated cell.

I factors are the factors which affect the initiation of differentiation, including the change of gene expression, environmental factors which affect genes etc. M factors are the factors that affect the cell membrane and change the membrane structure etc. In a case of sporulation in *Bacilli*, M factors contain the change in the cell which evoked calcium uptake. Additional factors essential for cell differentiation are supposed to exist. However, these two factors might be the primary factors at present.

SUMMARY

Although a wild-type strain of *B. megaterium* cultivated at 37°C was incapable of sporulation, a very small amount of calcium accumulation was observed in the delayed period of sporulation sequence, and the calcium accumulation level increased somewhat in the presence of added sodium dipicolinate. The incubation at 37°C seems to prevent the system of calcium accumulation being formed. Influence of the temperature-shift timing from 32 to 37°C on calcium accumulation during sporulation was examined, and it was shown that the temperature sensitive period was approximately at the end of growth. KCN inhibited calcium uptake as well as the formation of calcium accumulation system in asporogenous mutant 3-12 under sporulation condition, and chloramphenicol inhibited the formation of calcium accumulation system alone. Neither KCN nor chloramphenicol inhibited the decrease or degradation of calcium accumulation system in the mutant. In spite of the decrease of the amount of calcium accumulation, the calcium accumulation system and the stage of sporulation in the mutant were maintained after 64 hr in the medium containing 10% glycerol at −15°C. Factors necessary for sporulation and calcium accumulation were discussed.

Acknowledgement—I wish to thank Dr Charles Gilvarg for his generous support and kind discussion.

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INTERRELATIONSHIPS BETWEEN BODY WEIGHT, FOOD CONSUMPTION AND PLASMA THYROID HORMONE CONCENTRATION CYCLES IN THE WOODCHUCK, *MARMOTA MONAX*

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Abstract—1. The interrelationships between body weight, food consumption and plasma thyroid hormone concentrations were studied during the spring, summer and fall in two groups of woodchucks: those allowed to eat *ad libitum* and those kept at their March weight by food restriction.

2. The food consumption of the *ad libitum* fed control animals was greatest in April and May and then declined. Body weight in these animals peaked in July and then slowly declined during the rest of the study.

3. When the restricted animals were allowed to eat *ad libitum* in August they rapidly increased their food consumption and brought their body weight up to that of the controls in less than six weeks.

4. Plasma thyroxine (T_4) and triiodothyronine (T_3) concentrations decreased during the summer in the control animals and then rose again in the fall. The T_4 changes were blunted in the restricted animals.

5. T_3 concentrations decreased during the summer in the restricted animals and remained low until one month after they were allowed to eat *ad libitum*.

6. The low plasma concentrations of T_3 during the summer in the woodchuck may facilitate weight gain in preparation for hibernation.

INTRODUCTION

The woodchuck, like many other mammalian hibernators, has annual cycles of endocrine and metabolic function, appetite, physical activity and body weight (Young and Sims, 1979). The broad outlines of these cycles in hibernators have been known for many years (Kayser, 1961). One endocrine cycle which has been studied extensively is that of the thyroid gland (Hudson, 1981, for review). It has been found using morphologic methods that the follicular cells of the thyroid gland of the woodchuck are most active in the spring (Krupp *et al.*, 1977). The secretory activity then decreases during the summer and fall until the glands become quiescent during hibernation. Paradoxically, the circulating concentrations of triiodothyronine (T_3) are low during the weight gain period, possibly facilitating weight gain, but are highest during the fall and winter when the gland is quiescent (Young *et al.*, 1979). We have found in later studies that plasma thyroxine (T_4) concentrations are also elevated during the fall and winter (Young *et al.*, unpublished). Plasma T_4 and T_3 concentrations are also elevated during the fall and winter in the closely related Richardson's ground squirrel (Demeneix and Henderson, 1978).

In earlier studies it was found that the changes in serum thyroid hormone concentrations correlated better with the phase of the body weight cycle than with time of year (Young, 1975). Young animals would spontaneously achieve peak weight and peak thyroid hormone concentrations later in the summer and fall than would adult woodchucks because of the demands of growth as well as prehibernatory fattening. The present studies were designed to test the relationships between weight gain and plasma thyroid hormone concentrations by artificially postponing the weight gain phase of the cycle in adult woodchucks by food restriction.

MATERIALS AND METHODS

Four and five year old male woodchucks from the University of Massachusetts Medical School colony were used. They were housed in individual cages in a room kept at 21°C with a seasonally adjusted light cycle. They were fed Purina Rabbit Chow made by special order into blocks (Young and Sims, 1979) and were given water *ad libitum*.

The woodchucks were divided into two groups of seven animals. One group was fed the chow *ad libitum* and was allowed to gain weight normally. In mid March, starting three weeks after the termination of hibernation, the second group of woodchucks were individually given daily weighed amounts of food to maintain body weight at the mid-March level. The amount of food given the restricted animals was adjusted weekly. All woodchucks were weighed three times a week on consecutive days and an average weight was computed. Food consumption was measured weekly on two consecutive days and an average taken. Food consumption was calculated as the amount given minus the amount remaining (including crumbs and spillage) after 24 hr. On 10 August the restricted animals were again allowed to eat *ad libitum* until the end of the study.

At monthly intervals the woodchucks were tranquilized with ketamine hydrochloride and blood samples were taken by cardiac puncture into heparinized syringes. The plasma was separated and stored at −20°C until assayed. At the time of blood sampling the animals were given 1 cm^3 of a B vitamin and iron supplement intramuscularly to prevent anemia. Plasma T_4 and T_3 concentrations were determined by radioimmunoassay. Statistical analyses were performed with the Student's two-tailed *t*-test.

RESULTS

Body weight and food consumption data for the two groups of woodchucks are shown in Fig. 1. The *ad*

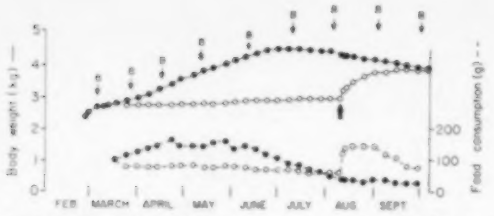

Fig. 1. Body weight (solid line) and food consumption (dashed line) of *ad libitum* fed (solid circles) and restricted (open circles) groups of woodchucks. Times where blood samples were obtained are marked with a B. The large arrow indicates the time when the restricted animals were allowed to eat *ad libitum*.

libitum fed animals gained weight steadily until their body weights reached a plateau in July. Body weights then decreased slowly until the end of the study. The average weight gained from mid-March until July in this group was approximately 1.75 kg. In contrast, the restricted animals gained an average of only 0.2 kg during the same period. When the restricted animals were allowed to eat *ad libitum* in August, they rapidly gained weight until, in little over a month, they weighed the same as the animals that had had *ad libitum* food during the entire study.

Food consumption by the *ad libitum* fed control woodchucks was greatest during April and May and then decreased steadily until August and September. The peak in food consumption occurred prior to the peak in body weight. The amount of food needed to maintain body weight in the restricted animals remained fairly steady until early June. At the time, even though the amount of food given was decreased, the animals started to gain weight slowly. When the restricted animals were given *ad libitum* food in August, they rapidly increased their food consumption and maintained it until they reached the same body weight as the control group.

Plasma T_4 concentrations in the *ad libitum* fed control woodchucks (Fig. 2) followed the expected pattern. The concentrations were high in the spring, lower in April, May and June, and then gradually rose during the rest of the study. Plasma T_4 concentrations in the restricted animals did not decrease as far in April and remained stable for the rest of the study. Plasma T_3 concentrations for the two groups are shown in Fig. 3. The plasma T_3 concentrations in both groups were quite high in the spring and lower during the summer. They then increased in the *ad libitum* fed control group. These values remained low in the restricted group until

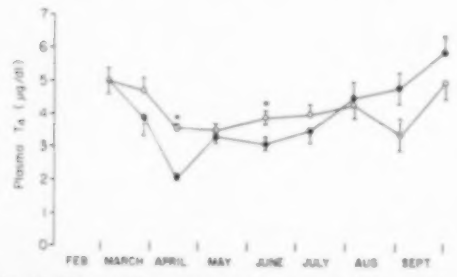

Fig. 2. Plasma T_4 concentration in *ad libitum* fed and restricted woodchucks. Points where the two groups are statistically different are marked with an asterisk.

Fig. 3. Plasma T_3 concentrations in *ad libitum* fed and restricted woodchucks. Symbols are the same as in Figs 1 and 2.

some time after the animals were given food *ad libitum*. The T_3 concentrations in the September plasma samples were still significantly lower in the restricted animals than in the control animals even though it had been a month since the animals were allowed to eat *ad libitum*.

DISCUSSION

The body weight and food consumption data from this experiment are similar to those described by the author and by others for woodchucks (Davis, 1967; Davis, 1971; Young, 1975) and ground squirrels (Pengelley and Fisher, 1963; Mrosovsky and Lang, 1971). In the control animals, food consumption reached its peak several weeks before body weight. After peak weight was attained, body weight in the *ad libitum* fed animals then declined gradually in the late summer and fall even though food was available and there was no opportunity for hibernation. In general, captive woodchucks reach their peak weight at an earlier date in each consecutive year (Young, 1975). This shift is a common observation in hibernators kept under constant lighting and temperature conditions and has been interpreted as evidence for an endogenous circannual rhythm of body weight with light as a Zeitgaber or entraining agent (Pengelley and Fisher, 1961; Pengelley and Asmundson, 1970). The animals in the present study were not kept under a set ratio of light and dark, however. An explanation other than a free-running, endogenous circannual rhythm would probably be more appropriate in this case.

Another possible explanation could be that of a sequence of linked stages (reproduction and weight loss, weight gain and glandular involution, hibernation and glandular regeneration), each one taking a certain amount of time to complete before leading into the next, with the last stage leading back to the first one again (Strumwasser *et al.*, 1967; Mrosovsky, 1967). If one of the stages were missing or curtailed, as reproduction is in most captive hibernators, the whole yearly cycle would be shortened.

Evidence against this theory in the present study is the weight gain of the restricted animals when they were allowed to eat *ad libitum*. Rather than taking several months as did the control animals to attain peak weight, the previously restricted animals weighed as much as the control group after only six weeks of *ad libitum* feeding. In addition, the body weights of the previously restricted animals did not reach the peak weight attained by the control animals but instead the weight of the controls at that time of the year (late September). Mrosovsky (Mrosovsky and Fisher, 1970; Mrosovsky, 1971) has postulated a "sliding set point"

to explain this type of finding in hibernators. The theory proposes that the set point for weight gradually increases during the summer with the animal regulating its food consumption, and thus its weight, so as not to be at variance with the "set". In the autumn, the set point gradually decreases and continues its downward trend throughout the winter. In the present study the sliding set point for body weight was the same in both groups, but it was not attainable by the restricted animals until after they were allowed to eat *ad libitum* in August and the peak set point for body weight had been passed. In a similar experiment Davis (1967) has reported that woodchucks kept on a restricted diet from January to June and then fed *ad libitum* adjusted their food consumption so that they reached peak weight at the same time as the controls.

The plasma T_4 and T_3 concentrations in the *ad libitum* fed control animals followed the expected pattern. They were elevated in the spring, decreased during the period of rapid weight gain in the summer and increased again in the fall. The low plasma T_4 and T_3 concentrations in the summer may have facilitated weight gain. The T_4 response was blunted in the restricted animals. It is of interest, however, that the plasma concentrations of T_3, the more active thyroid hormone, remained low in the restricted animals until some time after they were allowed to eat *ad libitum* and while they were gaining weight rapidly. Plasma T_3 concentrations did not rise in the restricted group until most of the weight gain had been achieved.

These data are in contrast to those found in rats and humans. In both rats and humans starvation or severe food restriction causes a decrease in plasma T_3 concentrations (Portnay *et al.*, 1974; Harris *et al.*, 1978; O'Brian *et al.*, 1980). Overfeeding in man causes an increase in plasma concentrations of T_3 (Danforth *et al.*, 1979). In the woodchuck, however, plasma T_3 concentrations are lowest during the time when food consumption is greatest and the most rapid weight gain is occurring. After the animals decrease their food intake so that they are actually losing weight, their plasma concentrations of T_3 rise.

The decrease in plasma T_4 and T_3 concentrations in late spring and summer parallel the general endocrine gland involution seen at the time in hibernators (Kayser, 1961). These changes are probably instrumental in the weight gain phase. The reasons for the increases in plasma T_4 and T_3 concentrations in the late summer and fall while the thyroid gland is nearly quiescent are not clear, but may be due to increases in serum thyroid hormone binding proteins (Young *et al.*, 1979). The elevations in plasma T_4 and T_3 may play a yet unknown role in the animals' survival during hibernation or may serve as a storage form of T_4 and T_3 for use after the animals arouse from hibernation in the spring. In any case, it can be seen from the data from this study that the summer weight gain and the changes in plasma T_4 and T_3 concentrations are interrelated. Artificially postponing the weight gain phase can postpone the rise in plasma T_3 concentrations seen in the late summer and fall, and plasma T_4 and T_3 concentrations remain low until the rapid weight gain phase of the yearly cycle is over.

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RENAL METABOLIC RESPONSE TO THERMAL STRESS BY THREE SPECIES OF GROUND SQUIRRELS*

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Abstract—1. Kidney mass, protein content and enzyme levels were ascertained for control, heat-acclimated and cold-acclimated *Spermophilus lateralis*, *Spermophilus tereticaudus* and *Amnospermophilus leucurus*.

2. Significant changes in kidney mass were observed in all three species.

3. In response to chronic temperature stress, *S. lateralis* showed a significantly smaller percent change in kidney oxidative metabolism than did *S. tereticaudus* and *A. leucurus*.

4. Kidney metabolic response is similar to that of liver in that it reflects an ecological basis for physiological differences.

INTRODUCTION

Adaptive regulatory and structural renal changes in mammals, especially rodents, inhabiting diverse, harsh environments have been areas of considerable interest to comparative physiologists and biochemists (Arine *et al.*, 1973; Bartholomew and Hudson, 1961; Bradley and Yousef, 1972; Chaffee *et al.*, 1963; Hudson, 1960, 1962; Inbar *et al.*, 1975; Schmidt-Nielson, 1964).

Extremely efficient renal functioning is a known characteristic of a number of desert-dwelling rodents: woodrats (*Neotoma* spp.), kangaroo rats (*Dipodomys* spp.), pocket mice (*Perognathus baileyi* and *P. penicillatus*), kangaroo mice (*Micodipodops pallidus*), jerboas (*Jaculus jaculus*), gerbils (*Gerbillus gerbillus*), grasshopper mice (*Onychomus torridus*) and squirrels (*Spermophilus nelisoni*, *Amnospermophilus leucurus*, *S. tereticaudus*) (Schmidt-Nelson, 1964). In general, diurnal desert rodents successfully conserve water through a variety of behavioral and physiological adaptations: they eat succulent foods; seek shade or burrows to unload heat; tolerate dehydration, and drink and process highly saline water; and maintain plasma volume and concentrate urine (Bartholomew and Hudson, 1959; Maxon and Morton, 1974; Bradley and Yousef, 1972). *S. leucurus* appears to have histological kidney function comparable to *Dipodomys*; both have long renal papilla that extend into the ureter (Hudson, 1962). A direct correlation has been suggested between capacity to concentrate urine and the length of the papilla (Schmidt-Nelson, 1958).

Previously, we reported our findings with regard to differences in enzymatic potential (Balcer *et al.*, 1976) and responses to chronic temperature stress (Balcer and Chaffee, 1981, 1982) among three species of squirrels—*S. lateralis* (hibernator, montane inhabitant), *S. tereticaudus* (estivator, low desert), and *A. leucurus* (non-hibernator, high desert). We found that the squirrels not only had real differences in oxidative enzymatic potential but also responded to acclimation with compensatory changes in brown adipose tissue (BAT) and liver tissue proteins, mass and enzymatic activities/levels. We noted distinct differences in BAT responses that correlated with genetic relationships (the hibernators vs the nonhibernators), whereas liver responses reflected ecological relationships (desert vs montane species). We were curious as to which trend other tissues, such as kidney, would follow.

In light of the low ratio of kidney-to-body weight, the kidney might be considered a relatively insignificant thermogenic organ. Arine *et al.* (1973) proposed, however, that the kidney's contribution to total heat production should not be overlooked because: (1) blood flow to the kidney is about 25% of the cardiac output, (2) kidney cortex is endowed with an active Na^{2+}/K^{+} ATPase (ADP-replenishing) system, and (3) heat production would warm the suprarenals and thus contribute to increased metabolic rate (in the cold).

MATERIALS AND METHODS

Squirrels were live-trapped in southern Nevada: *S. lateralis* in the Spring mountains at 2100 m elevation; *S. tereticaudus* and *A. leucurus* in the Los Vegas Valley at 650 m. The animals were caged individually on 12:12 hr light:dark cycles and fed Purina Lab Chow, sunflower seeds, fruits, carrots, and water *ad libitum*. Animals were killed within at least 6 weeks of acclimation to 24 ± 1°C (controls), 35 ± 1°C (heat), and 5 ± 1°C (cold).

Tissue removal and preparation of homogenates were described previously (Balcer *et al.*, 1976). Enzyme activities were measured polarographically at 37°C by the method of Chaffe *et al.* (1966). Protein content was determined by the Lowery spectrophotometric method.

All data were analyzed for significance by Student's *t*-test.

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Table 1. Intraspecific comparison of kidney weights* and kidney/body weight* ratio among temperature-acclimated *Spermophilus lateralis* (*S.l.*), *Spermophilus tereticaudus* (*S.t.*), and *Amnospermophilus leucurus* (*A.l.*)

	Heat (H)	Kidney weight (g) Control (Con)	Cold (C)	
S.l.	1.18 (5)†	0.870 (10)	1.63 (5)	C > Con‡ C > H‡ H > Con‡
S.t.	1.29 (6)	0.850 (16)	1.22 (3)	H > Con C > Con
A.l.	0.691 (6)	0.792 (23)	0.968 (5)	C > Con‡
		Kidney/body weight (‰)		
S.l.	0.567 (5)	0.487 (10)	1.03 (5)	C > H‡ C > Con‡
S.t.	0.443 (6)	0.832 (16)	0.827 (3)	C > H‡ Con > H‡
A.l.	0.635 (6)	0.834 (23)	0.959 (5)	C > H‡ Con > H‡

*Weight per kidney.
†( ) = Number of animals.
‡$P \leq 0.05$.

RESULTS

Absolute weight

Intraspecific comparisons are shown in Table 1. *A. leucurus* had a significant increase in kidney absolute weight after cold acclimation. *S. lateralis* and *S. tereticaudus* followed a similar trend of increased organ size after both cold- and heat-acclimation. There were no significant differences among controls; however, *S. lateralis* and *S. tereticaudus* had comparatively greater degrees of change in absolute kidney weight in both heat and the cold than did *A. leucurus* (Table 2).

Relative weights

Intraspecifically, a common trend of significantly increased relative kidney weight after cold acclimation was observed (Table 1). Unlike the two desert species, *S. lateralis* did not show reduced relative weight as a result of heat acclimation (Table 1). Although control *S. lateralis* had significantly lower relative kidney weight after cold acclimation, the percent change was twice that of the cold-acclimated desert species (Table 2). A significant difference was observed between the desert species: *S. tereticaudus* had smaller relative kidney weight after heat acclimation than did *A. leucurus*.

Table 2. Interspecific comparison of kidney weights* and kidney/body weight* ratio among temperature-acclimated *Spermophilus lateralis* (*S.l.*), *Spermophilus tereticaudus* (*S.t.*), and *Amnospermophilus leucurus* (*A.l.*)

	Cold (C)	Kidney weight (g) Control (Con)	Heat (H)
S.l.	187.58 (5)†	100 (10)	135.32 (5)
S.t.	143.0 (3)	100 (16)	151 (6)
A.l.	122.14 (5)	100 (23)	87.25 (6)
P	*S.l.* > *A.l.*‡ *S.t.* > *A.l.*‡		*S.l.* > *A.l.*‡ *S.t.* > *A.l.*‡
		Kidney/body weight (‰)	
S.l.	211.82 (5)	100 (10)	116.78 (5)
S.t.	99.4 (3)	100 (16)	53.1 (6)
A.l.	115.06 (5)	100 (23)	76.29 (6)
P	NSD		*A.l.* > *S.t.*‡

All data are normalized and expressed as percent of control, thus all control values are 100‰.
*Weight per kidney.
†( ) = Number of animals.
‡$P \leq 0.05$.
NSD = not significantly different.

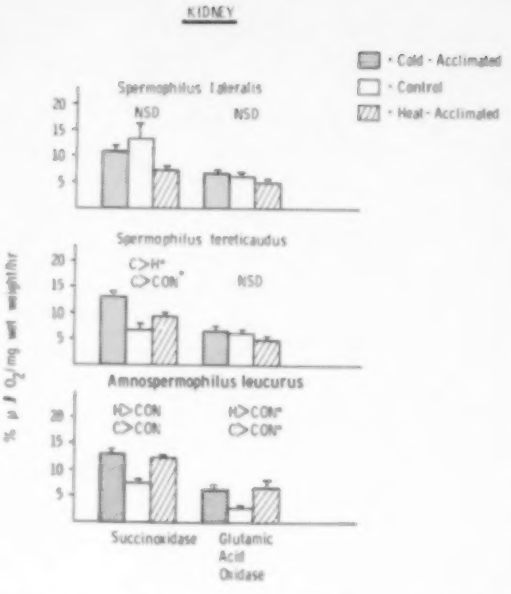

Fig. 1. Intraspecific comparison of oxidative metabolism (μg O_2/mg wet wt/hr $\pm$ SE) of kidney from control, cold-, and heat-acclimate *Spermophilus lateralis* (*S.l.*), *Spermophilus tereticaudus* (*S.t.*), and *Amnospermophilus leucurus* (*A.l.*) with different substrates. $P \leq 0.05$.

Kidney enzymatic activities (intraspecific)

Figure 1 shows intraspecific comparisons of kidney enzymatic activities. Cold-acclimated *S. tereticaudus* underwent significant increases in kidney succinate metabolism relative to control and heat-acclimated conspecifics. *A. leucurus* had higher specific activities in heat, as well as in the cold, compared with those of controls. *S. lateralis* did not respond with changes in kidney enzyme activities as a result of temperature acclimation.

Kidney enzymatic activities (interspecific)

Kidney interspecific comparisons of oxidative enzyme activities are shown in Fig. 2. Cold-acclimated *S. lateralis* had a significantly smaller change in kidney metabolic oxidation compared with that of the cold-acclimated desert species. Heat-acclimated *S. lateralis* also showed a significantly smaller percent change in specific activity than did either of the desert squirrels. With respect to glutamic acid oxidase activity, heat-acclimated *A. leucurus* had a comparatively greater activity than did *S. tereticaudus*.

Table 3. Protein content (mg/ml) ± SE of kidney from temperature-acclimated *Spermophilus lateralis* (*S.l.*), *Spermophilus tereticaudus* (*S.t.*), and *Amnospermophilus leucurus* (*A.l.*)

	Protein content of kidney			
	Cold (C)	Control (Con)	Heat (H)	
S.l.	34.65 ± 4.04 (5)*	22.58 ± 1.89 (10)	30.24 ± 3.52 (5)	H > Con†
				C > Con†
S.t.	25.69 ± 1.97 (3)	25.70 ± 4.66 (13)	28.0 ± 3.61 (6)	NSD‡
A.l.	33.64 ± 3.31 (4)	25.16 ± 1.91 (16)	36.744 ± 4.80 (6)	C > Con

*( ) = Number of animals.
†$P \leq 0.05$.
‡NSD = not significantly different.

Kidney protein

In heat- and cold-acclimated *S. lateralis*, protein levels changed significantly, relative to control levels. *A. leucurus* also had similar changes, although only heat-acclimated levels were significant. Protein content of *S. tereticaudus* did not change as the result of temperature acclimation.

DISCUSSION

Striking histological changes are known to occur in kidney tissue as the result of the temperature acclimation. In cold-acclimated animals the number of cells in the glomeruli increases, the number of multinucleate cells increases, and tubule size is more variable (Chaffee *et al.*, 1963; Roberts and Chaffee, 1975). With respect to weight changes in the cold, our data confirm similar findings in other species. Conversely, kidney hypotrophy has been described in heat-acclimated animals such as hamsters (Arine *et al.*, 1973), *Peromyscus maniculatus* (Roberts and Chaffee, 1975; Cassuto and Chaffee,1966; Chaffee *et al.*, 1969), and gerbils (Steffen and Roberts, 1977). The significant increase in kidney weight of *S. lateralis* compared with that of the desert species is interesting. *S. lateralis* might, in contrast to its desert relatives, perhaps compensate with a larger mass to deal effectively with the severe problems of water conservation, since it is not so well adapted to heat. The increase in tissue protein in heat-acclimated *S. lateralis* may be a simple reflection of greater growth requirements.

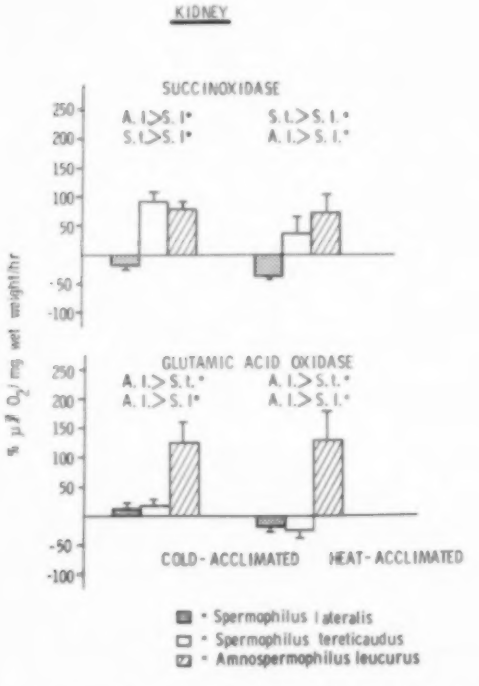

Fig. 2. Interspecific comparison of oxidative metabolism of kidney with different substrates of temperature-acclimated *Spermophilus lateralis* (*S.l.*), *Spermophilus tereticaudus* (*S.t.*), and *Amnospermophilus leucurus* (*A.l.*). $P \leq 0.05$. All data are normalized and expressed as percent change from control values, which are considered to be 100%.

The increased oxidative metabolism of kidney tissue observed in cold-acclimated desert squirrels compares with increased succinoxidase, DPNH-cytochrome-*c*-reductase, α-GP deydrogenase, and isocitric dehydrogenase activities found in the cold-acclimated hamster kidney (Chaffee *et al.*, 1963). Earlier studies have shown that total nitrogen, microsomal nitrogen, DNA content, and protein synthesis increased in kidneys of cold-acclimated animals (Chaffee *et al.*, 1963). In this study, both *S. lateralis* and *A. leucurus* responded to cold acclimation with increased protein levels. Although *S. tereticaudus* underwent no change in protein content, it maintained higher enzymatic activity in the cold. The fact that the desert squirrels had significantly smaller absolute and relative weights compared with those of *S. lateralis* and yet had significantly greater enzymatic activity suggests that cold acclimation was more of a stress to the desert species than to *S. lateralis*. The latter species was able to accommodate by simply increasing organ size (and thus the number of cells) without increasing cellular metabolic rates.

Although heat acclimation lowered metabolic activity in species such as hamsters and deer mice (Arine *et al.*, 1973; Cassuto *et al.*, 1970; Chaffee *et al.*, 1963, 1969; Inbar *et al.*, 1975; Roberts and Chaffee, 1976), among the squirrels in this study only *S. lateralis* followed this trend. Perhaps at 35°C the squirrels: (a) may not have been sufficiently heat-stressed to curtail oxidative metabolism; or (b) were still able to effectively dissipate metabolic kidney heat produced; or (c) required higher metabolic levels successfully to operate efficient renal systems because water loss has been shown to be related to heat loss and energy production. Production of urine is an important factor

in water balance, especially in desert animals (Bradley and Yousef, 1972). At an environmental ambient temperature (T_A), the urine of *A. leucurus* is 3.5 times more concentrated then when the animal maintained at 22°C. Furthermore, as T_A increases, electrolyte concentration increases (Hudson, 1960).

Interestingly, kidney respiration tends to be higher than that of the liver (Balcer *et al.*, 1976; Horwitz, 1974; Meyer and Morrison, 1960; Redmond and Layne, 1958). Cassuto *et al.* (1970) suggested that kidney cells are not as efficient as liver cells in reducing cellular heat production.

Horwitz (1964) suggested that higher kidney respiration is adaptive for the compensatory maintenance of electrolytic balance and excretion (both of which are affected by changes in body temperature). In addition, she hypothesized that the higher kidney metabolism (over a wide range of T_A) is adaptive in dealing with the increased production of end products from other tissues having elevated metabolism.

The extent of difference in glutamic acid oxidase levels between the two desert species may reflect other physiological and behavioral differences. *A. leucurus* is known to have a less efficient water turnover rate and a decreased ability to conserve renal water compared with *S. tereticaudus*. Yousef *et al.* (1974) suggested that differences in water turnover rate may relate to some evolutionary preadaptive feature of water regulatory mechanisms. *S. tereticaudus* is active during the hottest part of the day, during which T_A may reach 44°C; it lives in areas where soil temperatures reach 70°C and uses burrows to minimally avoid the high temperatures (Hudson, 1964: Hudson and Deavers, 1973; Vorhies, 1945). *A. leucurus* inhabits montane alluvial fans where T_A is not as extreme. It has a demonstrated lower heat tolerance than *S. tereticaudus*, remaning inactive and spending much of its time in underground burrows (Bartholomew and Hudson, 1961).

All three species tolerated the experimental thermal environment; some evidence of physiological compensation mechanisms (involving increases in the rate of heat loss by conductance and evaporative cooling) that were observed in the animals included: salivation and wetting of the fur, burrowing in cage litter, and stretching out on surface of the litter. The expense of heat dissipation by evaporative cooling mechanisms apparently was not costly enough (at 35 ± 1°C) to prevent survival.

The differences observed indicate that a physiological dissimilarity in coping with temperature stress exists between the montane *S. lateralis* and the two desert species. Thus, with respect to kidney metabolism, as is true of liver, ecological distribution appears to be a stronger unifying factor than genetic relatedness.

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COMPARISON OF THE INTRACELLULAR MEMBRANE POTENTIALS IN CRYSTALLINE LENSES OF VARIOUS FROGS

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Abstract—1. The intracellular membrane potentials were measured in the individual lens fibers of three kinds of frogs (*Rana catesbeiana*, *Rana pipiens japonica*, *Rana nigromata*) by a conventional glass microelectrode technique, in which the electrode was advanced to a depth of 200 μm from the anterior surface into the lens interior.

2. The maximum intracellular membrane potentials obtained in both anterior and posterior sides of all preparations were between −90 and −95 mV.

3. The measured potentials were nearly close to the E_K, and the membrane potentials in *in vitro* experiments correlated well with *in vivo* measurements.

4. Low membrane potentials reported previously in various frog lenses were discussed in comparison with the present results.

INTRODUCTION

Sperelakin and Potts (1959) have reported low intracellular membrane potentials of about −30 mV in the isolated bovine lens. Duncan *et al.* (1977) explained this low membrane potential resulting from a high sodium permeability in bovine lens, since this preparation still has a low internal sodium and a high internal potassium concentration even in such a low potential condition. Similarly, many electrophysiological data on amphibian lenses showed low membrane potentials as compared with theoretical values predicted by the Nernst equation based on their intracellular potassium concentrations determined in fresh lenses (Brindley, 1956; Duncan, 1969; Candia *et al.*, 1971; Rae, 1973; Paterson and Rae, 1974). Such low membrane potentials in frog lenses were also attributed to the relatively high permeabilities of the preparations for sodium and chloride (Delamere and Duncan, 1977). In addition, the membrane potential in the completely isolated frog lens was lower than that in the zonules-suspended lens (Rae, 1973).

From this laboratory, we also reported membrane potential of about −60 to −87 mV in the isolated *Rana catesbeiana* lens by using a conventional microelectrode technique (Murata *et al.*, 1974). Recently, however, we obtained high membrane potentials in the same preparation, in which the frog lenses were completely isolated by careful dissection under the dissection microscope and mounted in a glass chamber that could fix the isolated lens preparation without damage.

Therefore, the primary aim of this study was to discover the exact membrane potential of isolated frog lens in the intact condition, and so potential measurements were carried out on three different kinds of frogs. The second aim in the present study was to find out if there was a difference in membrane potential between *in vivo* and *in vitro* lens preparations.

MATERIALS AND METHODS

Materials, dissection and mounting

Specimens of three kinds of frogs (*Rana catesbeiana*, *Rana pipiens japonica* and *Rana nigromata*) were decapitated and the eyes were enucleated. In each case the posterior portion of the eye globe was dissected away and then the globe with lens suspended from zonules was pinned securely to the floor of a Lucite chamber. In this way, the *in situ* "posterior lens" preparation (Fig. 1A) was bathed on the posterior surface only with Ringer. The *in situ* "anterior lens" preparation (Fig. 1B) was prepared by removing both the cornea and iris. In this way only the anterior surface was perfused with Ringer. In *in vitro* experiments the lens was dissected free of surrounding tissues. Care was taken not to damage the lens. The "isolated lens" *in vitro* preparation was mounted in a special glass chamber with the anterior or posterior surface facing up and fixed to the glass cup covered with siliconized cloth and held in place by subatmospheric pressure (−100 to −130 mmHg) on the opposite surface (Fig. 1C). Tests showed that a pretty good seal was possible and no solution leaked from one side to the other.

Solutions

The Ringer solution used for bathing each frog lens had the following composition: NaCl 72.8 mM, KCl 2.5 mM, Ca-gluconate 2 mM, $MgSO_4$ 1.2 mM, Na_2HPO_4 2.9 mM, $NaHCO_3$ 26 mM and glucose 26 mM, and pH was adjusted by adding Tris-base and Hepes to 7.3 before use. The temperature of the solution was maintained at 18–20°C throughout all the experiments. The osmolality was about 230 mOsmol/l.

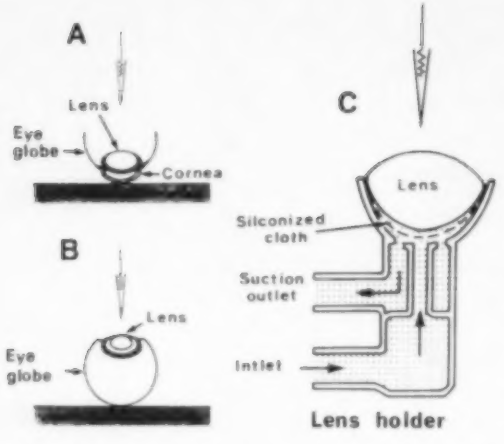

Fig. 1. (A) "Posterior lens" *in situ* preparation. (B) "Anterior lens" *in situ* preparation. In A and B preparations, Ringer was flowed over the entire preparation, in which one surface was selectively bathed in solution. This gives good contrast to the technique of the series of experiments of the suction apparatus (C). (C) Schematic illustration of the experimental apparatus for the potential measurements of crystalline lens fibers of isolated *in vitro* preparation. Glass chamber employed to selectively isolate either the anterior or posterior surface of the lens permitting independent perfusion of either surface with same or different artificial test solutions simultaneously. Different size glass chambers were used for different kind of frogs.

Membrane potential measurements

The intracellular potentials of individual lens fibers were measured with a conventional glass microelectrode filled with 3M KCl and having resistance of 15–30 MΩ in Ringer solution. The reference electrode was an Ag–AgCl electrode in 3M KCl solution which was connected via a Ringer–agar bridge to the bathing medium. Only microelectrodes with tip potentials of less than 5 mV in Ringer were selected for use. The potential difference between a microelectrode and a reference electrode was displayed on an oscilloscope through d.c. amplifiers with cathode follower and recorded continuously with a pen-writing recorder. The first electrical measurements were made at 40–60 min after the dissection.

The microelectrode was advanced perpendicularly to the plane of lens equator through the anterior or posterior surface into the lens interior by a stepping pulse-motor driven micromanipulator (Narishige SM-23) under a dissecting microscope. In most experiments the tip of the microelectrode was advanced to a depth of 200 μm from the lens surface since, in the topographical aspects of frog lens potential, the penetration of microelectrode to a depth of 100–200 μm gave a maximum resting potential of about −90 to −95 mV (Taura *et al.*, 1979). Repeated penetrations with a single microelectrode through the posterior surface was possible without tip breakage, but penetration of the anterior surface was more difficult and often damaged the microelectrode tip. Therefore, both the tip potential and resistance of microelectrode were always checked before and after anterior penetrations.

The numerical values are given as mean value ± 1 SD of the mean. Significance of the difference of means was decided by a Student's *t*-test.

RESULTS

Rana catesbeiana

Using the *in situ* "posterior lens" preparation with the posterior surface of the lens suspended upwards by its zonules, initial advances of the microelectrode produced deflections of small negative potential considered to result from a dimpling of the capsule by the electrode. Further advancement of the microelectrode resulted in a sudden, large negative shift measuring −91.3 ± 2.4 mV (mean ± SD, 50 fibers from 8 lenses). This usually occurred at a depth of about 100–200 μm and gave a maximal intracellular membrane potential of −95.3 ± 3.7 mV (42 fibers from 6 lenses). These maximal potentials decayed little with time. Measurement of membrane potential using the "anterior lens" preparation following removal of both the cornea and iris averaged −93.8 ± 4.3 mV (52 fibers of 6 lenses) at a depth of 200 μm. No significant differences were detected between the lens fibers of anterior and posterior sides in the two kinds of *in situ* lens preparations suspended from the zonules.

The *in vitro* "isolated lens" preparation experiments were carried out on lenses mounted in a glass chamber. In this way the potentials of anterior and posterior lens fibers could be measured in the same lens by alternately positioning either the posterior or anterior surface facing upwards. At depths of about 200 μm maximal potentials were measured and averaged −94.2 ± 3.7 mV (38 fibers from 11 lenses) in the anterior and −95.8 ± 4.3 mV (48 fibers) in the posterior. There was also no significant difference between the fibers of the other two sides. Furthermore, it is evident from the above results that there is no significant membrane potential difference between the zonules-attached *in situ* preparation and the isolated *in vitro* preparation.

Rana pipiens japonica *and* Rana nigromata

In a further series of experiments, two different species of Japanese frogs (*Rana pipiens japonica* and *Rana nigromata*) were subjected to a similar set of measurements. The membrane potentials in *in situ* "anterior" and "posterior" lens preparations at a depth of 200 μm from lens surface were −93.2 ± 2.8 mV (27 fibers from 5 lenses) and −92.3 ± 4.0 mV (22 fibers from 5 lenses) in *Rana pipiens japonica*, and −93.7 ± 3.1 mV (47 fibers from 5 lenses) and −92.9 ± 4.8 mV (20 fibers from 6 lenses) in *Rana nigromata*, respectively. Also, the membrane potentials of "anterior" and "posterior" surfaces in the isolated *in vitro* preparations averaged −93.2 ± 2.3 mV (30 fibers from 5 lenses) and −94.3 ± 3.0 mV (27 fibers from 6 lenses) in *Rana pipiens japonica*, and −95.0 ± 4.1 mV (40 fibers from 9 lenses) and −95.8 ± 4.1 mV (21 fibers from 6 lenses) in *Rana nigromata*. There was no significant potential differences between the anterior or posterior lens fibers in either the zonules-attached *in situ* preparations or the isolated *in vitro* preparations. These results confirm those obtained in the *Rana catesbeiana* lenses. The results are also summarized in Table 1 in comparison with those reported in various frog preparations.

Low-voltage compartments in the frog lens potential

Positive potential deflections (low-voltage compartments) from the resting potential level, which may be due to the glass microelectrode tip entering the extracellular spaces among lens fibers (Rae and Blankenship, 1973; Rae, 1974a, b), were observed in

Table 1. Comparison of the membrane potentials in various frog lenses measured *in situ* and *in vitro*

Author	Frog	$[K]_o$ (mM)	Membrane potential (mV) *in situ* (left in globe)	Membrane potential (mV) *in vitro* (dissected free)	*in vitro* condition
Brindley (1956)	—	2.5	A: −63 to −88 P: −79	A & P: −55 to −62	In chamber
Duncan (1969)	*Bufo bufo*	2.5	—	A & P: −63.5	In chamber
Candia *et al.* (1970)	*Bufo marinus*	2.3	—	A: −57 P: −31	Using type dividing chamber
Rae (1973)	*Rana pipiens*	2.5	A: −76.7 P: −72.5	A & P: −60	In chamber
Murata *et al.* (1974)	*Rana catesbeiana*	4.0	—	A & P: −60 to −87	Glass chamber
Delamere and Duncan (1977)	*Rana temporaria*	2.5	—	A & P: −63	In chamber
Preset experiments	*Rana catesbeiana*	2.5	A: −93.8 P: −95.3	A: −94.2 P: −95.8	Modified glass chamber
	Rana pipiens japonica	2.5	A: −93.2 P: −92.3	A: −93.2 P: −94.3	
	Rana nigromata	2.5	A: −93.7 P: −92.9	A: −95.0 P: −95.8	

$[K]_o$, the extracellular potassium concentration.
A and P indicate the lens fiber potential at anterior and posterior side, respectively.
Horizontal bars show no information or no measurement.

these frog lenses. The low-voltage compartments (−20 to −30 mV) were evident at depth between 600 and 1300 μm in *Rana catesbeiana* lenses and at depth between 300 and 900 μm in both *Rana pipiens japonica* and *Rana nigromata* lenses.

DISCUSSION

Anterior and posterior fibers of American common frog (*Rana pipiens*) lenses suspended from their zonules indicated the membrane potentials of −73 and −77 mV at 100–200 μm deep from each lens surface. Moreover, the lenses isolated completely from eye globe showed low potentials (about −60 mV) for both sides with no significant difference (Rae, 1973). Previous reports from this laboratory also showed low potentials of −60 to −87 mV at both surfaces of *Rana catesbeiana* lens (Murata *et al.*, 1974). Brindley (1956), Paterson and Rae (1974) and Delamere and Duncan (1977) reported such low potential values from frog lenses (Table 1). Similar results of low potential are observed in toad lens (*Bufo bufo*, *Bufo marinus*) (Duncan, 1969; Candia *et al.*, 1971). In the present experiments, however, the membrane potentials of anterior and posterior lens fibers measured at the depth of 200 μm from each lens surface were about −93 mV in both the American bullfrog (*Rana catesbeiana*) and two kinds of Japanese common frogs (*Rana pipiens japonica* and *Rana nigromata*), regardless of lens isolation or suspension from the zonules. Furthermore, the resting potentials measured in lenses of three different kinds of frogs in the present experiments are nearly close to the theoretical K^+ equilibrium potential (E_K = −91.6 and −95.3 mV) calculated from the Nernst equation by using the intracellular K^+ concentrations ($[K]_i$ = 94.9 and 110 mM), which were quoted from Table 3 in Delamere and Duncan (1977) and from intracellular K^+ activity in *Rana pipiens* fresh lens estimated by using a K^+ sensitive microelectrode (Paterson *et al.*, 1974), respectively. According to Rae (1974a), the increase of lens potential was accompanied by the elevation of electrode impedance and thus this large potential may be entirely artifactual. Therefore, in some experiments, we connected the glass microelectrode to a Wheatstone bridge circuit to record the potential from the lens fiber cells as well as to inject current pulses (Akaike *et al.*, 1976; Taura *et al.*, 1979). Consequently, the "input resistance" of lens fibers was hardly affected by the difference of the magnitude of resting potentials. In addition, when the potential profiles of individual lens fibers lying between the lens surface and its nucleus were examined in three kinds of frogs, the potential profiles were the same from lens to lens in each frog. Moreover, the contour maps of the isopotential lines of the *Rana catesbeiana* lens agreed well with the anatomical locations of individual lens fibers verified by Procion yellow dye injection (Hogan *et al.*, 1971; Taura *et al.*, 1979), suggesting that the high potentials in this paper are not the result of blocking of glass microelectrode tip. Therefore, the previous reports about low resting potentials published for various frog lenses may result from surgical or frictional damage to the lens surface in securing the lens to the floor of the bathing chamber (Rae, 1973). Similarly, damage may explain low potentials found in toad lenses with the use of the small Ussing-type glass holder tubing or large-tipped microelectrode (Candia *et al.*, 1970).

Utilizing a Ussing-type glass chamber for anterior–posterior isolation, Candia *et al.* (1970) placed a large-tipped microelectrode into the center of toad lens (*Bufo marinus*) and found a great difference between the potentials across the anterior (−57 mV) and posterior (−31 mV) surfaces, although the measured potentials were very low. Several studies also showed that, when the anterior surface of frog, toad and bovine lenses was electrically isolated in a divided chamber (Candia *et al.*,

1970; Kinsey and McGrady, 1971; Delamere and Duncan, 1979) or by immersing in liquid paraffin (Duncan *et al.*, 1977), asymmetrical potentials (anterior face positive) existed. In the present *in situ* and *in vitro* experiments, however, there was no asymmetry potentials between the anterior and posterior lens fibers. This result agrees with Duncan *et al.*'s (1977) and Brindley's (1965) previous observations measured in *in situ* preparation. It seems that the lens constitutes an isopotential system since a short circuit occurs through the region of the suspensory ligaments *in situ* preparations or the lens capsule (Takeguchi and Nakagaki, 1970) and deficient seal between the lens and the chamber in *in vitro* preparations.

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LUNG RESPIRATION, SOMATIC ACTIVITY AND GAS METABOLISM IN EMBRYONIC CHICKS PREVENTED FROM HATCHING BY THIOUREA

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Abstract—1. Treating chick embryos with high doses of thiourea (32.8 μmol) on day 17 of incubation resulted in prevention of hatching and of active breathing.

2. Furthermore, thiourea also prevented the increase of O_2 consumption and the marked increase of somatic activity associated with the final hatching act.

3. These findings provide evidence for the importance of active breathing in the prenatal period to initiate pipping and hatching of the avian embryo.

INTRODUCTION

Extensive studies on metabolism of the chick embryo (Romijin and Lokhorst, 1956, 1960; Visschedijk, 1968; Rahn *et al.*, 1974) showed that its metabolic rate increased during incubation and tended to reach a plateau just prior to pipping of the shell. O_2 needed for metabolic processes is supplied via the pores in the eggshell and the vessels of the chorioallantoic membrane underlying the eggshell (chorioallantoic respiration).

The factors regulating gas exchange between the internal and external environments have been extensively studied by Rahn and his coworkers (Wangensteen *et al.*, 1970/71; Wangensteen and Rahn, 1970/71; Paganelli *et al.*, 1975). According to these studies, gas exchange is limited by diffusion through the pores of the eggshell. Obviously, its conductance is high enough to permit the metabolic rate established at the plateau prior to hatching. However, an additional amount of O_2 may be needed for the exertion of hatching, which cannot be delivered by the pores because of the fixed eggshell conductance. It is at this developmental stage that the embryo penetrates the air cell with its beak (internal pipping) and begins pulmonary ventilation. Lung respiration offers a chance to improve the O_2 supply, but may not be very effective as long as gas exchange is limited by the eggshell conductance. As shown by Visschedijk (1968), only a slight increase of O_2 consumption is observed during the paranatal period, lasting from the beginning of active breathing to pipping the eggshell (external pipping), whereas external pipping is followed by a marked increase of O_2 consumption.

It has been assumed (Visschedijk, 1968) that towards the end of incubation hypoxia occurs, from which the embryo tries to escape by pipping the eggshell. Hypoxia may be caused mainly by the active breathing of the embryo in the air cell on one hand and by the limited gas exchange through the eggshell on the other hand. So lung ventilation may act as a stimulus for the hatching process.

Romanoff and Laufer (1976) and Balaban and Hill (1971) reported that chick embryos treated with high doses of thiourea are prevented from hatching. If lung respiration is involved in the initiation of hatching, then it is reasonable to assume that lung respiration is affected in these embryos. Therefore, in the experiments reported here, the respiratory pattern and gas metabolism of thiourea-treated embryos were investigated. Since the possibility exists that somatic activity is influenced by the lung respiration, the activity pattern of the embryo was measured in addition to respiratory activity.

MATERIALS AND METHODS

Treatment of the eggs

Fertile eggs from Leghorn hens, obtained from a commercial supplier, were incubated at a temperature of 37–38°C and a relative humidity of 63–67%. In order to treat embryonic chicks with thiourea, a small hole was made in the eggshell with the needle of a syringe and 32.8 μmol thiourea was injected into the eggs, avoiding injection of the embryo. The hole was covered with glue after treatment. Eggs were not turned from day 17 onwards. In this way interference with the activity pattern should be avoided.

Measurements of O_2 and CO_2 tensions in the air cell

To measure partial pressures of O_2 and CO_2 in the air cell a method described by Tazawa *et al.* (1980) was used. A hole was drilled through the blunt end of the eggshell and a syringe was fixed above it, so that air in the syringe was in direct contact with gas in the air cell. By analysing the partial pressures in the syringe, after equilibration between air cell and syringe was established, it was possible to determine the O_2 and CO_2 concentrations in the air cell. It takes about 2 hr to equilibrate partial pressures of O_2 and CO_2 between the air cell and the syringe (Tazawa *et al.*, 1980). In our experiments the syringes were connected to the air cell at least 3 hr before analysing the air samples in a micro-Scholander apparatus.

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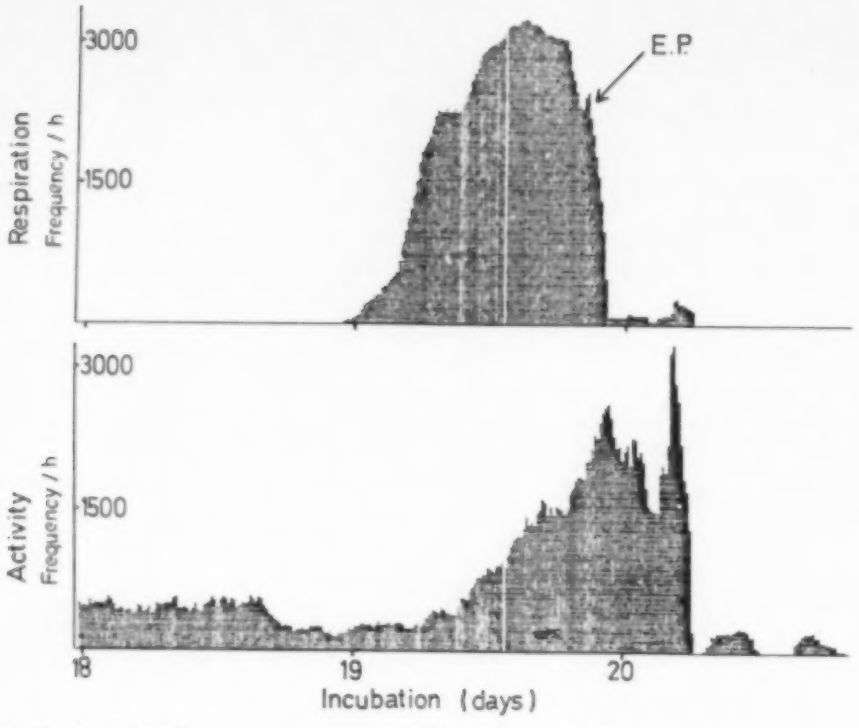

Fig. 1. Activity and respiratory pattern of embryonic chicks at the end of incubation. The sharp decrease of activity frequencies at day 20 of incubation is caused by external pipping, as pointed out in the method section. E.P. = external pipping.

Measurement of O_2 consumption

For measuring O_2 consumption a modified Scholander respirometer (Scholander and Edwards, 1942) was used. An incubation chamber, containing the incubated egg and 20% KOH, which is needed to absorb CO_2 produced by the embryo during incubation, and another chamber used for compensation, were placed in a waterbath at incubation temperature. Each chamber was connected to the arms of a U-tube. At the beginning of the experiment the incubation chamber was gassed with O_2 and then occluded with an O_2 filled syringe. O_2 consumption by the embryo reduces the gas pressure in the incubation chamber and unbalances the manometer. The original fluid level is restored by injecting O_2 from the syringe into the incubation chamber. The volume of O_2 injected in this way can be followed from the calibration of the syringe. It equals the amout of O_2 consumed by the embryo during a given time interval when connected to S.T.P.D.

Recording respiratory and somatic movements

Respiratory movements were recorded from the pressure changes caused by active breathing in the air cell. A small hole was drilled through the eggshell overlying the air cell and a plastic tube was inserted. The tube was connected to a piezoelectric crystal, which transformed the pressure changes in the air cell into corresponding electrical signals. These were amplified and continuously recorded by a computer system. The method allows recording of respiratory frequencies during the paranatal period. External pipping reduces the pressure changes within the egg after which respiratory frequency can no longer be recorded (Fig. 1).

For monitoring somatic movements of the embryo, the egg was suspended in a holder. A small wire, which was connected to a piezoelectric crystal, contacted the egg at the upper curvature. The forces caused at this point of the eggshell by the movements of the embryo were imparted to the piezoelectric crystal and transformed into corresponding electrical signals. These were amplified and continuously monitored by a computer system. Technical details of respiration and motility registration were reported in previous papers (Kugler *et al.*, 1982; Wittmann *et al.*, 1983).

RESULTS AND DISCUSSION

The normal chick embryo displays a high activity during incubation (Fig. 1). Around day 19 of incubation a short period of reduced activity is observed, which is followed by a very drastic increase of activity leading to pipping and finally to hatching. During this phase of reduced activity, which presumably signals the attainment of hatching position, lung ventilation is initiated. Frequency of respiratory movements increases in parallel with activity (Fig. 1). As shown in previous experiments (Wittmann *et al.*, 1983), activity and respiratory patterns can also be observed when hatching is accelerated by L-thyroxine and retarded by thiourea respectively. Lung ventilation and the marked increase of motility starts earlier than normal under the influence of L-thyroxine and later than normal under the influence of thiourea.

When 32.8 μmol thiourea was injected into eggs instead of 13.1 μmol as in previous experiments (Wittmann *et al.*, 1983), most of the embryos (70–80%) were unable to hatch, although almost 40% survived until day 25 or longer. Pipping of the eggshell took place in a few cases, but internal pipping was not as a rule prevented by thiourea.

Furthermore, a delayed growth rate was observed under the influence of thiourea (Table 1).

Table 1. The influence of thiourea on the growth rate of embryonic chicks

	Incubation (days) 19	20
Control		
Body wt (g)	27.4 ± 2.38	30.9 ± 2.49
N	7	12
Thiourea-treated		
Body wt (g)	23.6 ± 2.06*	25.2 ± 2.27†
N	7	14

In the experimental group 32.8 μmol thiourea was injected into the eggs on day 17 of incubation. All values in this and in the following tables represent means ± SD. Significance of changes was tested by Student's *t*-test. *$P < 0.05$, †$P < 0.001$. N = number of embryos.

Injecting high doses of thiourea resulted in marked changes of the respiratory and motility pattern of the embryo. As can be seen in Fig. 2, embryos which were prevented from hatching did not display a significant increase in activity towards the end of incubation. In addition, initiation of lung respiration was prevented by thiourea.

In some embryos treated with thiourea lung ventilation was initiated, but respiratory frequency decreased after several hours (Fig. 3). In these cases there was also a significant increase of activity towards the end of incubation, but compared with controls this increase in frequency was small. In controls somatic activity increased by a factor of 5–6, whereas in treated embryos (Fig. 3) a factor of about 2 was recorded. As in the case of lung respiration, activity also decreased several hours later.

So far, the results indicate an intimate relationship between activity and respiration at the end of incubation. In all our measurements lung ventilation started before the onset of intensified movement activity, which was followed by hatching. On the other hand, when lung respiration was totally prevented, the drastic enhancement of activity was also prevented. These findings suggest that the increased capacity to take up O_2 by lung ventilation mediates enhanced somatic activity which itself is needed in order to break the eggshell and to escape from the egg. From this point of view it is reasonable to assume that hatching is prevented in thiourea-treated embryos as no adequate O_2 supply for the exertion of the hatching act is established because of the failure of lung ventilation.

If this is true, then one would expect the metabolic rate of chicks which are prevented from hatching to be lower than in embryos displaying normal lung respiration. In the following experiments, therefore, we measured the O_2 consumption of controls and of thiourea-treated chicks.

As can be seen in Table 2, O_2 consumption is increased towards the end of incubation. Such an increase was not observed when hatching was prevented with thiourea.

As pointed out above, active breathing may be involved in causing hypoxia, which should act as a stimulus for pipping the eggshell. On the other hand, hypoxia should not occur in those cases where lung ventilation is suppressed. In a final experiment, therefore, we measured the time-course of changes in O_2- and CO_2-concentrations in the air cell of controls and thiourea-treated embryos. As demonstrated in Table 3, on day 20 of incubation in controls O_2 percentages are lower and CO_2 percentages are higher than in thiourea-treated embryos. However, there is no significant difference in the respiratory quotient (RQ) between control and experimental.

From these findings one can draw the conclusion that in embryos with undeveloped lung respiration gas exchange does not exceed the conductance of the eggshell and, therefore, hypoxia does not occur. The embyro is not stimulated to pip the eggshell. Obviously, gas exchange via chorioallantoic respiration is enough to sustain the O_2 supply for metabolic requirements for a period much longer than geneti-

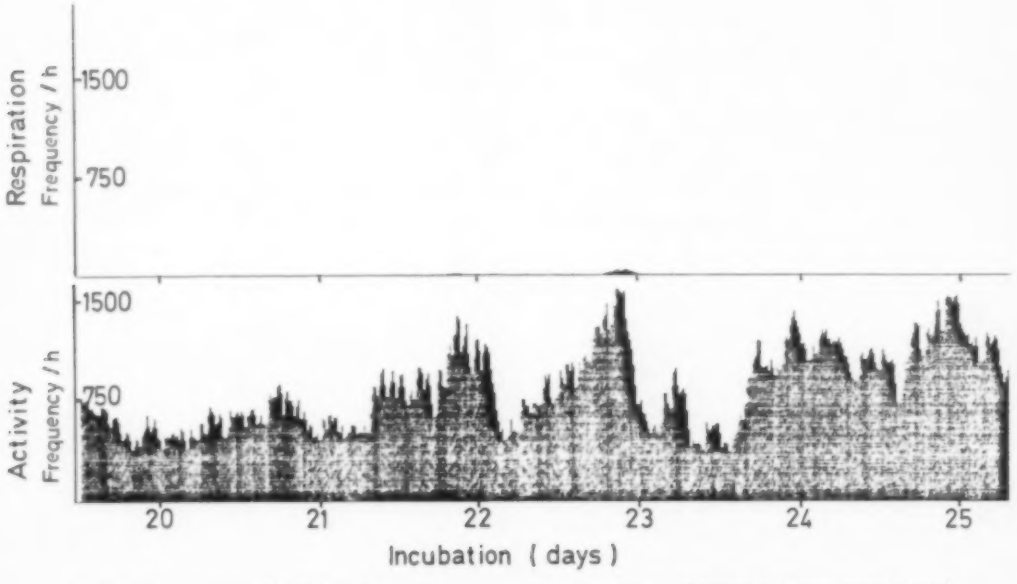

Fig. 2. Activity pattern of a chick embryo with suppressed lung respiration. Thiourea (32.8 μmol) was injected into the egg on day 17 of incubation.

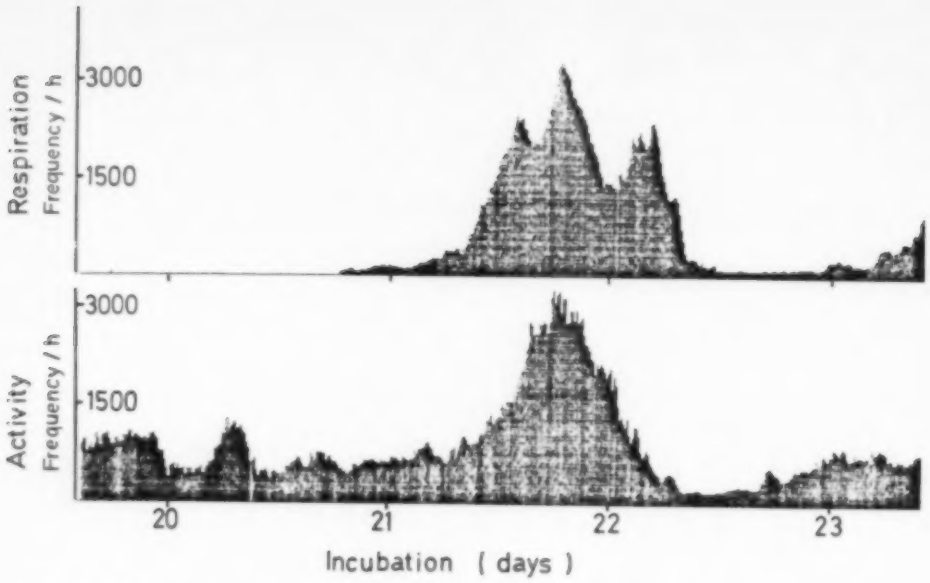

Fig. 3. Activity and respiratory pattern of a chick embryo with incomplete lung respiration. Thiourea (32.8 μmol) was injected into the egg on day 17 of incubation.

cally provided. In our experiments, thiourea-treated embryos survived until day 25 and in a few cases until day 26 without pipping the eggshell. On the other hand, if lung respiration is not initiated, the additional O_2 needed for the exertions of the hatching act cannot be provided, as pointed out above. So, it seems very likely that suppression of lung respiration is the primary factor preventing thiourea-treated embryos from hatching.

However, Balaban and Hill (1971) reported that under the influence of thiourea hatching is prevented, even if breathing is initiated. From this observation

Table 2. The influence of thiourea on O_2 consumption of the embryonic chicks beginning with day 17

	Incubation (days)					
	17	18	19	20	21	22–25
Control						
O_2 consumption (ml/hr STPD)	17.1 ± 3.5	18.4 ± 1.4	18.0 ± 2.1	19.8 ± 3.3	31.1 ± 7.2*	
N	7	9	9	16	11	
Thiourea-treated						
O_2 consumption (ml/hr STPD)		18.9 ± 2.9	18.8 ± 3.4	19.4 ± 3.7	19.0 ± 3.74	19.4 ± 5.3
N		8	9	12	29	21

In the experimental group 32.8 μmol thiourea was injected into the eggs on day 17 of incubation. *$P < 0.001$. *N* = number of embryos.

Table 3. The influence of thiourea on O_2 and CO_2 in the air cell

		Incubation (days)				
		18	19	20	21	22–25
Control	O_2	18.22 ± 0.76	17.51 ± 0.52	16.21 ± 1.02		
	N	13	12	7		
	CO_2	2.38 ± 0.63	3.29 ± 0.45	4.16 ± 0.72		
	RQ	0.69	0.75	0.69		
Thiourea	O_2	18.53 ± 0.50	18.20 ± 0.60	18.35 ± 1.08*	18.37 ± 1.22	18.67 ± 0.67
	N	7	11	10	18	13
	CO_2	2.39 ± 0.46	3.09 ± 0.89	2.59 ± 0.90*	2.21 ± 0.90	2.39 ± 0.78
	N	7	14	10	23	15
	RQ	0.78	0.88	0.78	0.67	0.82

In the experimental group 32.8 μmol thiourea was injected into the eggs on day 17 of incubation. RQ = respiratory quotient. *$P < 0.001$. *N* = number of eggs.

they concluded that active breathing is necessary for hatching, but its occurrence is not sufficient for the appearance of the hatched state. As shown in the experiments reported here, in some cases hatching was also prevented, although lung ventilation was initiated (Fig. 3). However, in these chicks breathing ceased after a while. So, gas exchange via lung respiration may be not sufficient for those animals to hatch.

In this context it should be mentioned that Balaban and Hill (1971) recorded breathing by direct observation of the respiratory movements. In embryonic chicks, however, respiratory movements are observed before true breathing begins (Kuo and Shen, 1937; Vince *et al.*, 1975). In our experiments lung respiration was registered by the pressure changes caused by breathing. This is a more reliable method for recording an effective gas exchange via the lungs.

No information can be presented on the mechanism suppressing lung ventilation. As thiourea antagonizes the metabolic effects of thyroid hormones, it is very likely that a hypothyroid status is involved. The function of thyroid hormones with respect to lung development is not understood. However, data are available which indicate a stimulatory effect of thyroid hormones on the formation of surfactant (Blackburn *et al.*, 1972; Wu *et al.*, 1973; Ballard *et al.*, 1980). On the basis of our preliminary results, however, it seems possible that circulation of the lungs is affected under these conditions. In contrast to controls, on day 20 of incubation no increase of haemoglobin concentrations in lung was observed, when hatching was prevented by thiourea.

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EFFECT OF DIETARY PROTEINS ON THE FREE AMINO ACID CONTENT IN RAINBOW TROUT (*SALMO GAIRDNERI* RICH.) MUSCLES

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Abstract—1. Groups of rainbow trout were fed for 12 weeks on diets containing different levels of the poultry-by-product meal. Concentration of free amino acids was measured in the latero-dorsal muscle.
2. Despite similar levels of nutritive substances and exogenous amino acids, the diets were characterized by different nutritive values for trout.
3. Patterns of free amino acids in the muscle tissue were different in particular fish groups.

INTRODUCTION

Levels of free amino acids in fish tissues have been studied not only with regard to amino acid metabolism, but also in order to determine amino acid requirements of fish (Kaushik, 1979; Robinson *et al.*, 1980; Wilson *et al.*, 1977). The relationship between content of exogenous amino acids in fish diet and free amino acid concentration in fish tissues, determined for trout (Kaushik and Luquet, 1979; Nose, 1972; Schlisio and Nicolai, 1978), carp (Dabrowski, 1982; Nose *et al.*, 1978; Plakas *et al.*, 1980), roach and rudd (Knapp and Wieser, 1981) was taken advantage of by Murai *et al.* (1982) for an assessment of the quality of dietary proteins. The latter authors proposed to use the balance of exogenous amino acids in the plasma (BEAAp) as an index of the diet quality. It was found that this index significantly correlated with the PER values, fish growth and food utilization.

The present studies were undertaken in order to assess the effect of various levels of poultry-by-product meal, supplemented with synthetic amino acids, in the fish diet upon the level of free amino acids in rainbow trout muscles.

MATERIALS AND METHODS

Rainbow trout (*Salmo gairdneri* Rich) yearlings, of average individual weight of 34 ± 7 g, were used in the present study. Experiments were carried out in aquaria supplied with lake water, at continuous aeration and regulated temperature. Thermal and oxygen conditions were similar in each aquarium; water temperature amounted to 15°C, oxygen content to 7.5 mg O_2/l. Fish were fed the experimental diets three times daily, for 12 weeks. Analyses were made at the end of this period. Daily feed rations were established according to the standards given by Leitritz (1969).

The diets differed as regards the source of protein (Table 1) and the content of exogenous amino acids (Table 2). In diets 2 and 3, 23% and 50% respectively of the fish meal protein were replaced by poultry-by-product protein. In diets 4, 5 and 6 all fish meal was substituted with poultry-by-product meal. In order to balance the proteins, exogenous amino acids were added to diets 5 and 6. The experimental diets covered quantitative requirements of salmonid fish for nutritive substances and exogenous amino acids (Mertz, 1969), with the exception of sulphuric amino acids. Crude protein content in the diets amounted to 42.6–43.6%, and crude fat to 9.7–10.7% on dry matter basis. The diets were not supplemented with sulphuric amino acids because cystine content in the poultry-by-product meal was higher than in the fish meal.

Content of free amino acids was determined in latero-dorsal muscle samples. Ten fish were removed from each experimental group 14 hr after the last feeding. The muscle was homogenized in 5% sulphosalicylic acid, centrifuged, and the supernatant (after readjusting pH to 2.2 with citrate buffer) was analysed using an amino acid analyser, model Joel JLC 6AH (Japan).

RESULTS

The type of the dietary proteins noticeably affected utilization of the experimental diets (Table 3) and concentration of free amino acids in rainbow trout muscles (Table 4). Substitution of the fish meal with poultry-by-product meal resulted in slower fish growth and worse utilization of the diets (Wajno and Dabrowska, in press).

The concentration of exogenous amino acids in the muscles of fish fed diets 3, 4, 5 and 6 (in which 50% and 100% of the fish meal were replaced by poultry-by-product meal) was significantly lower (415.4–588.4 mg/100 g dry muscle) than in the fish fed control diet (717.3 mg/100 g dry muscle) containing fish meal as the main source of proteins. Similarly, the EAA/NEAA ratio was significantly lower in the muscles of experimental fish compared with the control. On the other hand, the total content of free amino acids did not differ significantly.

Replacement of total fish meal protein by poultry-by-product meal in diet 4 noticeably decreased the concentration of free histidine, lysine and sulphuric amino acids in fish muscles, whereas there was a significant increase of valine, threonine and concentration compared with the fish fed the control diet.

Addition of synthetic amino acids to diets 5 and 6 resulted in an increase of their concentration in the fish muscles. When 1.5% lysine was added to diet 5, the level of this amino acid was significantly higher (279 mg/100 g dry muscle) than in the muscles of

Table 1. Composition of the experimental diets given to rainbow trout (%)

Constituents	Diet number 1	2	3	4	5	6
Fish meal	30	22.5	15	—	—	—
Poultry-by-product meal	—	6	12	24.2	24.2	24.2
Meat and bone meal	10	10	10	10	10	10
Yeast	10	10	10	10	10	10
Soya bean meal	10	10	10	10	10	10
Wheat meal	14	14.5	15.5	17.8	16.3	15.6
Barley meal	19	19	19	19	19	19
Fish oil	5	6	6.5	7	7	7
Vitamin mixture*	2	2	2	2	2	2
L-lysine	—	—	—	—	1.5	1.5
DL-histidine	—	—	—	—	—	0.5
L-tryptophan	—	—	—	—	—	0.2

*Vit. A 400 000 I.U., vit. D_3 50 000 I.U., vit. E 10 g, vit. K_3 1.5 g, vit. B_1 1.5 g, vit. B_2 4.0 g, vit. B_6 1.5 g, vit. B_{12} 3 mg, Ca-pantothenate 4.0 g, folic acid 0.4 g, nicotic acid 8.0 g, inositol 30 g, biotine 44 mg, choline chloride 50 g, vit. C 24 g, BHT 3 g, per 1 kg of vitamin mixture.

Table 2. Amino acid composition of the experimental diets (g/16 g N)

	Diet number 1	2	3	4	5	6
Phenylalanine	4.04	3.83	4.23	4.10	4.06	4.57
Tyrosine	2.64	2.41	2.61	2.30	2.29	3.09
Leucine	7.41	6.71	7.40	6.93	6.90	7.75
Isoleucine	4.09	3.74	4.16	4.08	3.98	4.27
Methionine	1.71	0.90	1.10	0.46	0.34	0.74
Valine	4.92	4.86	5.52	5.67	5.72	5.95
Cystine	0.77	1.24	1.78	2.47	2.45	2.62
Threonine	4.05	3.67	4.07	3.81	3.64	3.77
Arginine	5.90	5.54	5.84	5.99	5.69	6.11
Histidine	2.62	2.10	1.92	1.23	1.15	2.39
Lysine	7.00	5.62	5.32	3.54	6.42	6.62
Tryptophan	0.91	0.71	0.54	0.30	0.30	0.48

fish from group 4 (126.5 mg/100 g dry muscle) and control fish (205 mg/100 g dry muscle). Addition of histidine to diet 6 increased the level of free histidine in fish muscles compared to that in the muscles of fish from group 4, but it was still significantly lower (228 mg/100 g dry muscle) than in the control fish (415 mg/100 g dry muscle).

Trace amounts of free cystine in the muscles of fish fed diets 4, 5 and 6, notwithstanding a cystine content three times greater and the low coefficient of digestibility of this amino acid (Dabrowski and Dabrowska, 1981), suggest that cystine originating from poultry-by-product meal was badly utilized by rainbow trout.

DISCUSSION

The level of free amino acids in the tissues not only depends on the type and content of proteins in the diet, deficiency or excess of specific amino acids (Harper, 1967; Nose *et al.*, 1978), digestibility or amino acid availability, but also varies during and after food consumption (Dabrowski, 1981, 1982;

Table 3. Evaluation of the experimental diets

	Diet number 1	2	3	4	5	6
Average individual weight gain (g)	46.4	73.3	65.9	50.1	45.8	40.6
Conversion factor	1.27	1.28	1.36	1.71	1.77	1.96
PER	1.96	1.96	1.86	1.47	1.41	1.27

Table 4. Concentrations of free amino acids in the muscle of rainbow trout (mg/100 g dry muscle, mean ± SD)

	Group of fish 1	2	3	4	5	6
Phenylalanine	8.1 ± 2.3^{abc}	6.2 ± 0.7^{c}	7.6 ± 1.4^{abc}	7.3 ± 1.6^{bc}	9.9 ± 1.4^{ab}	10.2 ± 0.9^{a}
Tyrosine	6.4 ± 0.3^{ab}	8.0 ± 1.2^{a}	4.3 ± 1.8^{bc}	5.8 ± 0.8^{ab}	6.8 ± 0.8^{ab}	3.2 ± 2.2^{c}
Leucine	9.5 ± 0.3^{c}	16.4 ± 0.4^{a}	11.4 ± 1.5^{cd}	12.3 ± 1.0^{bc}	14.6 ± 3.0^{ab}	12.6 ± 1.0^{bc}
Isoleucine	5.9 ± 0.6^{b}	10.9 ± 0.3^{ab}	12.9 ± 8.3^{a}	8.6 ± 0.5^{ab}	9.9 ± 2.1^{ab}	9.2 ± 0.6^{ab}
Methionine	10.6 ± 2.0^{ab}	14.6 ± 2.4^{a}	13.2 ± 2.7^{a}	6.5 ± 2.0^{b}	9.8 ± 3.2^{ab}	8.0 ± 1.9^{b}
Valine	13.5 ± 0.9^{b}	22.1 ± 1.1^{a}	17.6 ± 1.0^{ab}	21.8 ± 3.1^{a}	23.1 ± 3.9^{a}	20.9 ± 4.3^{a}
Cystine	5.0 ± 0.2^{a}	6.0 ± 3.1^{a}	1.1 ± 0.4^{b}	tr.	tr.	tr.
Alanine	107.7 ± 23.2^{c}	116.7 ± 14.8^{c}	122.8 ± 17.9^{bc}	146.2 ± 4.5^{ab}	152.9 ± 5.9	131.6 ± 3.8^{abc}
Glycine	487.0 ± 56.2^{ab}	363.3 ± 57.6^{b}	479.5 ± 93.7^{ab}	555.4 ± 50.8^{a}	496.3 ± 117.2^{ab}	459.1 ± 98.5^{ab}
Proline	28.8 ± 22.6^{a}	50.5 ± 23.2^{a}	62.2 ± 6.6^{a}	60.0 ± 25.9^{a}	50.1 ± 13.2^{a}	35.8 ± 13.8^{a}
Glutamic acid	40.2 ± 5.4^{d}	52.2 ± 1.2^{cd}	46.9 ± 3.4^{cd}	71.8 ± 8.5^{b}	102.1 ± 9.7^{a}	56.4 ± 13.2^{c}
Serine	30.6 ± 8.8^{c}	38.9 ± 8.5^{c}	56.5 ± 3.5^{b}	69.0 ± 4.2^{b}	92.5 ± 18.9^{a}	65.5 ± 0.6^{b}
Treonine	38.9 ± 9.1^{b}	50.4 ± 15.1^{ab}	40.6 ± 3.4^{b}	64.4 ± 9.8^{a}	64.0 ± 13.2^{a}	48.8 ± 8.7^{ab}
Aspartic acid	10.0 ± 3.7^{b}	14.2 ± 6.3^{ab}	10.4 ± 3.7^{b}	21.8 ± 4.4^{a}	20.9 ± 1.9^{a}	19.1 ± 2.4^{a}
Arginine	4.3 ± 0.1^{c}	7.9 ± 0.2^{bc}	8.9 ± 0.0^{b}	12.5 ± 1.8^{b}	26.5 ± 5.4^{a}	10.2 ± 1.8^{b}
Histidine	415.0 ± 65.5^{a}	351.8 ± 14.0^{a}	24.7 ± 34.0^{b}	149.7 ± 50.9^{c}	144.8 ± 69.3^{c}	228.0 ± 29.6^{bc}
Lysine	205.0 ± 61.0^{b}	206.6 ± 22.6^{b}	165.9 ± 14.6^{bc}	126.5 ± 8.6^{c}	279.0 ± 66.2^{a}	191.7 ± 18.6^{bc}
Sum of FAA	1421.6^{a}	1336.7^{a}	1309.1^{a}	1339.6^{a}	1503.2^{a}	1310.3^{a}
Sum of EAA	717.3^{a}	700.9^{a}	530.8^{b}	415.4^{c}	588.4^{b}	542.8^{b}
EAA/NEAA ratio	1.02^{a}	1.10^{a}	0.68^{b}	0.45^{c}	0.64^{b}	0.71^{b}

Duncan's multiple range test was applied and groups followed by the same superscript letters are not significantly different ($P < 0.05$).

Nose, 1972; Kaushik, 1979; Murai *et al.*, 1982). It seems that muscle sampling 14 hr after fish feeding, as suggested by Nose (1972) and Kaushik and Luquet (1977), is quite adequate for testing of the hypothesis posed in the introduction.

The relationship between the level of free amino acids in the tissues and their content in the diet is without doubt. Nevertheless, it seems that the suggestion by Fujita *et al.* (1981) that free amino acids do not directly reflect the amino acid composition or level of ingested protein, but rather indirectly point to total metabolism pattern in the tissues and their response to the quality and quantity of proteins consumed, is quite true. This effect is shown by different levels of free arginine and threonine in the muscles of particular trout groups, although the content of these amino acids in the diets was similar. On the other hand, concentrations of valine in diets 3, 4, 5 and 6 were higher than in diets 1 and 2 and may have led to increased concentrations of this amino acid in the muscles of fish given these diets. The leucine/isoleucine ratio in the diets was similar, amounting to 1.7–1.8, i.e. within the range recommended for single-stomached animals. Still, there were some differences in the level of these amino acids in the muscles of particular fish groups.

In nutritional studies, and most of all in cases of determining nutritive value of unconventional protein sources in fish diets, estimation of free amino acids in the tissues may constitute a good index of protein quality. Furthermore, it seems that the BEAAp index, proposed by Murai *et al.* (1982) for carp, and calculated in a similar way to the EAA index, is not suitable for trout. BEAAp values, calculated for trout fed diets 1–6, amounted to 100, 130, 128, 179, 167 and 142 respectively, and did not agree with the results of earlier studies on fish growth and utilization of the diets (Table 3).

In the present study, significantly lower EAA/NEAA values and sums of EAA for muscles of fish fed diets 3, 4, 5 and 6, compared with muscles of fish fed diets 1 and 2, were confirmed by lower values of PER and conversion factors for these diets. Nose *et al.* (1978) found that deficiency of exogenous amino acids in a carp diet resulted in lower concentration of these amino acids in the muscles, and considerable lowering of the EAA/NEAA ratio.

The results suggest that in fishes fed diets 3, 4, 5 and 6, the lower rate of protein synthesis was caused mainly by the lower availability of amino acids in the poultry-by-product meal, and by an imbalance of some exogenous amino acids in these diets. Significantly decreased levels of free histidine and lysine in the muscles of fish from group 4 confirm the suggested deficiency of these amino acids in diet 4, and justify their addition to diets 5 and 6. Concentration of these amino acids in fish muscles in groups 5 and 6 increased, as did the sums of EAA and the values of EAA/NEAA. These results and the studies of amino acid absorption in trout (Dabrowski and Dabrowska, 1981) show that free amino acids added to the diets in order to improve nutritive quality of the protein were not effectively utilized by trout. Notwithstanding this, some authors have improved the effects of fish feeding by supplementing the diets with proper levels of limiting amino acids (Nose, 1974; Dabrowska and Wojno, 1977; Ramsey and Ketola, 1975; Reinitz *et al.*, 1978; Smith, 1977; Steffens and Albrecht, 1976).

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THE ACTIVITY OF THE HYPOTHALAMO-NEUROHYPOPHYSIS DURING REHYDRATION FOLLOWING WATER DEPRIVATION IN THE GERBIL (*MERIONES UNGUICULATUS*) AND THE LABORATORY RAT (*RATTUS NORVEGICUS*)

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Abstract—1. Water deprivation in gerbils and rats for 5 and 3 days respectively resulted in the same degree of dehydration of the animals and similar depletion of the neurohypophyseal vasopressin stores.

2. Following access to drinking water, the vasopressin stores were replenished in the gerbil and rat in 2 and 6 days respectively.

3. It was suggested that the quicker restoration of the store in the gerbil was due to the greater ability to synthesize vasopressin and to continuing high activity of the neurosecretory cells until the stores were replete.

INTRODUCTION

The level of the hypothalamo-neurohypophyseal hormones in the neural lobe of the pituitary is considerably reduced during water deprivation (Hild and Zetler, 1953; Diamond, 1956; Lederis, 1962). This depletion presumably results from a greater release of the hormones into the circulation than can be replaced by synthesis in the hypothalamic magnocellular neurons. Donaldson and Edwards (1981) showed that the depletion was much less in the gerbil, a species adapted to the semi-desert Steppes of Northern Asia, than that observed by Jones and Pickering (1969) and Legros and Dreifuss (1975) in the mesically-adapted rat. It was suggested that the smaller fall in the hormone store of vasopressin in the gerbil could indicate a greater ability to synthesize this hormone (Donaldson and Edwards, 1981).

To examine this hypothesis rats and gerbils were deprived of water for 3 and 5 days respectively to deplete the neural lobe of vasopressin to approximately the same extent. The animals were then allowed access to drinking water again and the rate of repletion of the neural lobe stores of vasopressin was determined as a measure of synthesis of vasopressin in the two species. In addition the size of the supraoptic neurosecretory nerve cell bodies was measured during the various states of hydration. Changes in the dimensions of these cells and their inclusions are likely to reflect changes in the synthesis of the posterior pituitary hormones (Enestrom, 1967; Rechardt, 1969; Watt, 1970; Hatton and Walters, 1973; Morris and Dyball, 1974).

MATERIALS AND METHODS

Male and female rats of the DA strain (*Rattus norvegicus*) weighing between 172 and 240 g were housed singly in metabolism cages while female and male gerbils (*Meriones unguiculatus*) weighing between 54 and 78 g were housed in pairs of the same sex. All animals were maintained at 25 ± 0.5°C at a relative humidity of 33 ± 7% on a 12 hr photoperiod. They were fed Rat and Mouse No. 3 diet (Special Diet Services, Witham, Essex) *ad libitum*.

Control animals with free access to water were placed in the metabolism cages for 5 days (gerbils) and 3 or 10 days (rats) while water-deprived animals spent 5 days (gerbils) and 3 days (rats) in these cages. Other gerbils and rats which had been water-deprived for the above times were allowed access to water for varying times.

During the time spent in these cages the urine produced was collected under paraffin oil to prevent evaporation and its amount and osmolality determined each day.

At the end of the periods described above the animals were anaesthetized between 1000 and 1200 hr with 60 mg sodium pentabarbitone (Nembutal)/kg body weight and were decapitated. Blood was collected from the neck and its haematocrit determined. After centrifugation the osmolality of the plasma was determined using a Fiske QF osmometer. The vasopressin content of the neural lobe of the pituitary was determined by the rat blood pressure technique of Dekanski (1952) using Pitressin (a gift from Parke, Davis and Co., Pontypool) as a standard.

Hypothalamic tissue was fixed overnight in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4 and was embedded in methacrylate resin. Transverse sections (3 μm thick) were stained with 0.1% toluidine blue for 30 sec. Fifty perikarya of the supraoptic nucleus and their inclusions from each hypothalamus were drawn onto standard white paper using a Leitz tracing device and a SM Lux microscope giving a total magnification of 1250 times. Only cells with visible nucleoli were drawn. The cross sectional areas of the cell bodies and their nuclei were determined by weighing.

The significance of all results was analysed by Student's *t*-test.

RESULTS

Gerbil

Following 5 days of water deprivation the vasopressin content of the neural lobe had fallen by 55% ($P < 0.01$) (Fig. 1). There were significant increases of 13.4 and 4.6% in the haematocrit and plasma osmolality (both $P < 0.01$) and falls in the body weight (18.5%) and urine volume while the urine osmolality

increased (all $P < 0.001$) (Table 1). There was a significant increase in the size of the supraoptic nerve cell bodies ($P < 0.05$), this resulting from an increase in the cytoplasmic ($P < 0.01$) but not nuclear area.

Following access to drinking water for 1 day the haematocrit, plasma osmolality and body weight had returned to the control level (Table 1). The vasopressin store was still depleted at this time ($P < 0.001$) (Fig. 1) and the urine volume was still considerably lower ($P < 0.01$) and its osmolality still elevated ($P < 0.001$) compared to control animals. By the second day of rehydration the neural lobe vasopressin content and urine volume were not significantly different from the control values though the urine osmolality remained elevated on the second and third days of rehydration ($P < 0.02$, $P < 0.05$ respectively). The cell bodies of the supraoptic neurons were still larger on day one of rehydration ($P < 0.05$) due, as before, to the increased cytoplasmic area ($P < 0.01$), but had returned to control size by the second day of rehydration.

Rat

The results of the control animals after 3 and 10 days in the metabolism cages were pooled to cover the experimental period. After 3 days of water deprivation the vasopressin level in the neural lobe had fallen by 42% (Fig. 1), the haematocrit and plasma osmolality increasing significantly by 14.1 and 4.5% respectively with the body weight falling by 15.8% (all $P < 0.001$) (Table 2). The amount of urine produced by the third day of deprivation was significantly reduced and it was of a higher osmolality (both $P < 0.001$).

After 1 day of rehydration the haematocrit and urine volume and osmolality were back to control levels, the plasma osmolality falling to just below the control value and remaining lower for the duration of the experiment with the exception of day 6 (days 1 and 2, $P < 0.05$; days 4 and 7, $P < 0.02$). The body weight and neural lobe vasopressin store were not restored to control values until days 4 and 6 of rehydration respectively.

DISCUSSION

It would appear that, following water deprivation for 3 and 5 days in the rat and gerbil respectively, the extent of dehydration was approximately the same in the two species as indicated by similar changes in the body weight, vasopressin level in the neural lobe, haematocrit and plasma osmolality. An increase was found in the size of the supraoptic nerve cell bodies of the hypothalamus of the gerbil, resulting from an increase in the area of the cytoplasm. An increase in the cytoplasmic area has also been shown in the rat (Morris and Dyball, 1974), reflecting increased synthesis of vasopressin in these cells during dehydration.

In both species the blood appears to have returned quickly to normal by the end of the first day of rehydration at least in terms of the haematocrit and plasma osmolality. The return of the plasma osmotic pressure to normal within 24 hr has also been reported in the rat by Rougon-Rapuzzi *et al.* (1978). The gerbil was still producing a small volume of more concentrated urine on day one of rehydration despite free access to drinking water, whereas the rat produced urine of a similar volume and concentration to control animals. This strategy might help explain the much quicker return of the body weight to normal in the gerbil (1 day in the gerbil compared to 4 days in the rat). The continued antidiuresis in the gerbil suggests that a greater release of vasopressin is taking place than normally occurs when water is freely available. In spite of this increased release of hormone the stores of vasopressin in the neural lobe were replenished in only 2 days, this coinciding with a return of the cell area of the supraoptic perikarya to the control value.

In the rat the stores of vasopressin were not completely replaced until the sixth day of rehydration and it would appear from the urine concentration and volume that only basal amounts of vasopressin were being released into the blood. Indeed, Dyball and Pountney (1973) have shown that the firing of supraoptic neurons had decreased back to control rates by the second day of rehydration suggesting a return to control levels of vasopressin release at this time. A return of the vasopressin stores to control levels has also been shown after 5 days in normal rats of the Brattleboro strain (Moses and Miller, 1970) and rats of the Sprague–Dawley strain (Rougon-

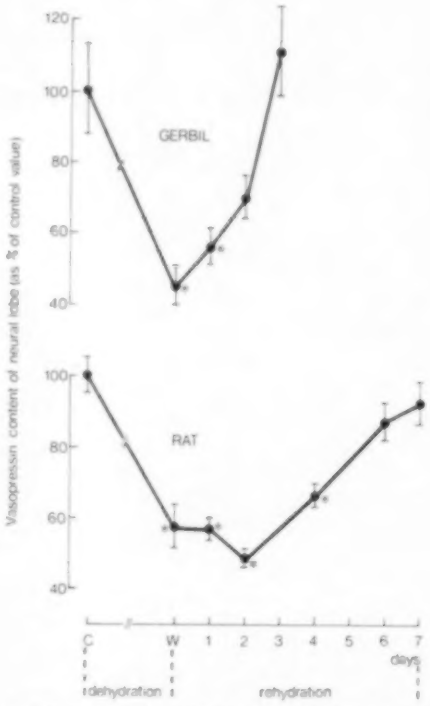

Fig. 1. The vasopressin content of the pituitary neural lobe of control (C) and water-deprived (W) gerbils and rats, and following rehydration. The gerbils and rats were deprived of drinking water for 5 and 3 days respectively. Each point represents the mean ± SE for six animals except for control rats where $N = 12$. Asterisked points are significantly different from their respective control value. The control levels of vasopressin in the gerbil and rat in absolute terms were 388.3 ± 49.4 and 332.4 ± 16.9 mU/100 g initial body weight respectively.

Table 1. The body weight, blood, urine and size of the supraoptic perikarya in control, dehydrated and rehydrated gerbils

	Control	Water-deprived	Days of rehydration		
			1	2	3
Body weight (% of initial b.w.)	101.2±0.9 (21)	81.5±0.6 (26)d	99.5±1.4 (6)	99.9±1.6 (6)	99.5±0.8 (6)
Haematocrit (%)	44.1±1.0 (6)	50.0±1.1 (6)c	41.8±0.7 (6)	43.8±0.7 (6)	44.6±0.4 (6)
Plasma osmolality (mosmol/kg)	341.2±2.5 (6)	356.8±2.6 (6)c	345.2±2.2 (6)	339.7±2.6 (6)	344.7±1.5 (6)
Urine volume (g/100g b.w.)	4.95±0.87 (8)	0.26±0.06 (6)d	1.66±0.19 (9)c	3.03±0.47 (6)	3.98±0.97 (3)
Urine osmolality (mosmol/kg)	1475±173 (11)	4692±182 (11)d	2963±137 (9)d	2095±152 (6)b	2085±216 (3)a
Supraoptic perikarya					
cell area	204.4±8.4 (11)	232.7±8.7 (11)a	225.5±4.2 (6)a	240.6±19.0 (6)	224.6±22.4 (6)
nuclear area	65.1±2.6 (11)	67.8±2.3 (11)	63.1 ±1.7 (6)	68.9±3.7 (6)	65.8±5.7 (6)
cytoplasmic area	139.3±6.1 (11)	164.9±6.5 (11)c	162.4 ±4.1 (6)c	171.7±16.0 (6)	158.9±16.9 (6)

The cell, nuclear and cytoplasmic areas of the supraoptic perikarya are given in μm^2. Results are expressed as mean ± SE with number of animals in parentheses; b.w. = body weight. Significant changes from control values are shown as: a, $P < 0.05$; b, $P < 0.02$; c, $P < 0.01$; and d, $P < 0.001$.

Table 2. The body weight, blood and urine of control, dehydrated and rehydrated rats

	Control	Water-deprived	Days of rehydration				
			1	2	4	6	7
Body weight (% of initial b.w)	100.4±1.3	84.2±0.3c	93.1±0.9c	94.3±0.6c	96.6±1.3	100.7±1.2	99.9±0.8
Haematocrit (%)	41.4±0.4	46.8±0.7c	41.7±0.5	41.2±0.7	40.9±0.6	40.8±0.6	40.9±0.6
Plasma osmolality (mosmol/kg)	319.0±1.5	333.3±1.3c	315.2±1.2a	315.2±0.8a	314.2±1.3b	316.2±1.0	314.2±1.3b
Urine volume (g/100g b.w.)	2.96±0.43	0.40±0.05c	2.43±0.24	2.45±0.35	2.50±0.16	2.86±0.39	4.06±0.50
Urine osmolality (mosmol/kg)	1883±105	2948±127c	1663±183	2039±184	2192±155	2022±312	1405±215

The results are expressed as mean ± SE for six animals except for control values where $N = 12$; b.w. = body weight. Significant changes from control values are shown as: a, $P < 0.05$; b, $P < 0.02$; and c, $P < 0.001$.

Rapuzzi *et al.*, 1978). However, Young and Van Dyke (1968) and Livingston (1973) have reported that the levels of vasopressin and oxytocin respectively did not return to control levels until day 14 of rehydration. The rather different recovery times reported could reflect the treatment used in causing dehydration (imbibition of 2% saline or water deprivation), the duration of the treatment (3–7 days), the posterior pituitary hormone measured (vasopressin or oxytocin) or possibly to the different strains of rat that were used (Wistar, Sprague–Dawley, DA or Brattleboro). Significant differences have been shown in the size of the vasopressin stores of different strains of rat (Edwards, 1980) and it is possible that there are also differences in the rate of hormone synthesis during rehydration.

During the period in which vasopressin stores are being replenished in the rat, the neurosecretory cell bodies of the supraoptic nucleus have been variously reported to be still enlarged even after 10 days of rehydration (Hatton and Walters, 1973) or back to normal by 2 days of rehydration (Morris and Dyball, 1974). In the latter case synthesis would have returned to normal a number of days before stores were completely replenished, indicating the lack of a feedback system whereby synthesis of hormone is regulated by the level of neural lobe stores. Whether the cells are synthesizing vasopressin at normal or increased rates the restorative process is still much slower than in the gerbil.

The increased efficiency with which the gerbil replenishes its vasopressin stores could be due to a greater ability to synthesize vasopressin compared with the rat as already suggested by Donaldson and Edwards (1981) who showed that water deprivation of both species for an identical period resulted in a much greater fall in the vasopressin stores in the rat. During rehydration the greater efficiency may also be due to the continuing high rate of synthesis in the gerbil until the stores are replete, while in the rat synthesis may have returned to control levels some days before the stores are back to normal. Such adaptations would be of considerable value to the gerbil, living in an environment where water is scarce.

This power of replenishment has been shown during longer-term water deprivation in three desert species. In two species of spiny mice, *Acomys cahirinus* and *A. russatus* (Castel and Abraham, 1972) and in the spinifex hopping mouse, *Notomys alexis* (Bridges and James, 1982) depletion of the neural lobe stores of vasopressin occurs initially but gradually the stores are replenished even though water deprivation continues.

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ON THE ENERGY FUEL IN VOLES DURING THEIR STARVATION

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Abstract—1. Starved voles derive energy predominantly from endogenous carbohydrates, unlike small laboratorial rodents which use free fatty acids as fuel during their fasting.
2. This seems the reason why deep hypoglycemia is developed in fasting voles.
3. The rate of gluconeogenesis in starved voles is rather high while oxidation of carbohydrates in that state is more rapid than their formation.
4. Utilization of fat in fasted voles is relatively small. Voles can die after starvation still having a lot of fats in their bodies.
5. Oxygen consumption in starved voles decreases to a small extent.

INTRODUCTION

The patterns of the substrates used in bioenergetic processes in starved mammals, including man, have been widely studied by many investigators (Benedict and Fox, 1934; Cahill *et al.*, 1966, 1968; Babirak *et al.*, 1974; Cuendet *et al.*, 1975; Brady and Armstrong, 1977; Parilla, 1978; Goodman and Ruderman, 1980; Goodman *et al.*, 1980; see also Abderhalden, 1934; Newsholm and Start, 1973). The kind of fuel used in small wild rodents during starvation is not known.

It has been shown recently that starvation in Microtinae is accompanied by a profound hypoglycemia which is much more pronounced than that in Muridae and other mammalian species (Mosin, 1982). The reason for those differences is unclear. Two things seemed to be responsible for deep hypoglycemia in starved Microtinae: (i) the consumption of endogenous carbohydrates is rather large and/or (ii) gluconeogenesis in these species is weak. Other factors such as hormonal disturbances probably are not important because the kinetics of hormonal action in that state does not favour hypoglycemia (Cahill *et al.*, 1966, 1968; Owen *et al.*, 1969; Cuendet *et al.*, 1975; Xavier *et al.*, 1976; Goodman and Ruderman, 1980; Goodman *et al.*, 1980).

In the present work, studying the respiratory exchange ratio (RER) in starved voles, we study the contribution of carbohydrates and fats in their energy metabolism and the reason for deep hypoglycemia in fasting animals.

MATERIALS AND METHODS

The adult males of voles (Microtinae) *Clethrionomys rutilus* and *Clethrionomys rufocanus* were used in the experimental studies in 3–5 months after trapping near Magadan. During that time they were kept in the vivarium individually caged into "terrarium cells" supplied with a nest box and wheel for running. The animals were fed meal (grain mixture, fruits, vegetables and vitamins) and given water *ad libitum*. The light schedule was natural and the temperature was 18°C. Three days before the experiment the voles were moved into transparent plastic chamber (20 × 20 × 20 cm) for adaptation, with the same facilities as in "terrarium cells". At the beginning of the experiment food was removed for the fasted group, the chamber was made airtight and thermostatically controlled at 19°C. The air in chamber was periodically renewed and the quantities of oxygen and carbon dioxide in the air samples derived from the chamber were determined manometrically. The data obtained were normalized.

RESULTS

Most of the starved animals died after 20–26 hr of fasting. This agrees with our previous observations (Mosin, 1982). The voles were usually active until death. They ran along the wheel and tried to find food. RER in fed *Cl. rutilus* and *Cl. rufocanus* was rather high. It oscillated mostly in a range from 0.94–1.03. Sometimes the RER fell, usually between 06.00 and 08.00 a.m. and between 06.30 and 08.30 p.m. (Fig. 1a, c). In the starved voles RER is also relatively high; however, it falls to a low value more frequently. This fall does not depend on the time of day, but on the time of starvation. For example, RER decreased after 5–6 hr of starvation, whenever the experiments were begun. Afterwards it significantly rose again and then oscillated until 20–22 hr of fasting. At the end of the day of starvation RER usually dropped (Fig. 1b, d).

The oxygen consumption in starved voles tends to decrease compared with that in non-starved voles. At rest in both fasted and fed voles it was 2.2–2.6 ml/g/hr and 2.0–2.2 ml/g/hr for *Cl. rutilus* and *Cl. rufocanus* (Table 1). During exercise oxygen consumption increased, the extent depending on the rate of running; on average by 3–5 times, at most by 10 times (Table 1).

DISCUSSION

As index of patterns of bioenergetic substrates RER ($V_{CO_2}:V_{O_2}$) has been used by many authors (Benedict and Fox, 1934; Consolazio *et al.*, 1963; see also Abderhalden, 1934). It was established that when only free fatty acid or carbohydrates are oxidized, RER is equal to 0.707 or 1.0 respectively. The increase of RER to 0.707–0.8 may be due to the oxidation of both carbohydrates and proteins (amino acids), although

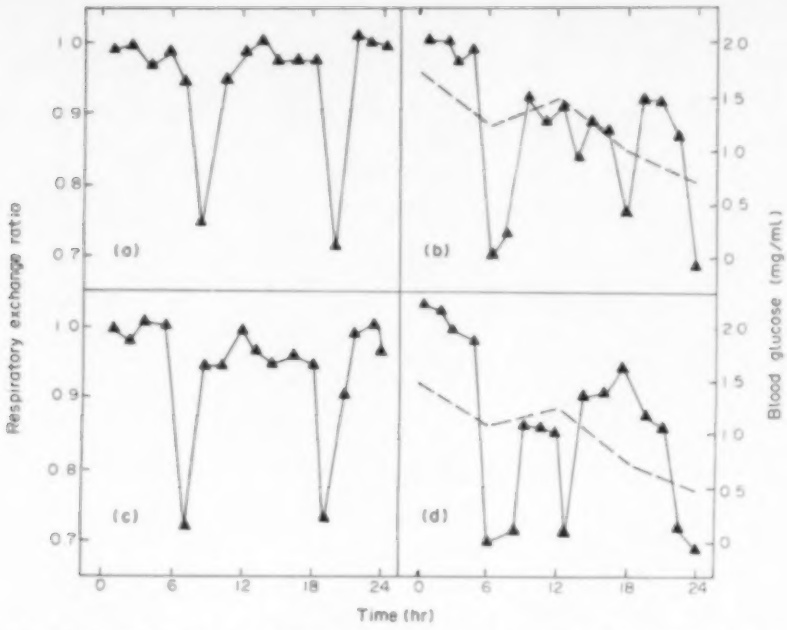

Fig. 1. Respiratory exchanger ratio in starved (b, d) and fed (a, c) voles of *Cl. rutilus* (a, b) and *Cl. rufocanus* (c, d). Results of typical experiments. Broken line is the blood glucose concentration (data from the previous work, Mosin, 1982).

the relative contribution of the latter is rather small (Benedict and Fox, 1934; Wahren *et al.*, 1971). The rise of RER higher than 0.8 is only due to the oxidation of carbohydrates.

In fed voles the RER is large (Fig. 1a, c), in agreement with the observations of others (see Koshtoyanz, 1950), which show that RER in fed herbivores is equal to 1.0, whereas RER in flesh-eaters is rather small (near 0.8). The decrease of RER in fed voles in the morning and in the evening (Fig. 1a, c) seems to be a manifestation of diurnal biorhythm.

In starved hebivores the RER at the beginning of the post-absorptive period is also large. Then it gradually declines to about 0.7–0.8 when endogenous carbohydrate reserves are exhausted, and stays at the same level throughout the period of starvation (Benedict and Fox, 1934). RER in voles during the first 2–3 hr of fasting is near 1.0; by 5–6 hr of starvation it decreases to 0.7. Afterwards RER rises again and remains at about 0.85–0.90 during the period of fasting (Fig. 1b, d). This means that both oxidation of carbohydrates and gluconeogenesis in these species progresses rapidly. Glucose production, however, is not always sufficient to cover its oxidation. This follows from the facts that: (i) RER drops (in an irregular manner) from time to time during starvation, and (ii) the blood glucose concentration is progressively decreased after 12 hr of starvation (Fig. 1b, d). At the end of the day of fasting, RER, as a rule, is very low. The glucose level in blood at that time in some voles falls to 0.1 mg/ml or below. The deep hypoglycemia seems to be the reason for death because of the many systems, particularly the central nervous system, which need glucose for their nutrition (Krebs, 1971; Cahill *et al.*, 1968).

The physiological peculiarity on starved voles, therefore, is the relatively large consumption of carbohydrates by their bioenergetic processes, whereas other laboratory animals and man can successfully use free fatty acids and ketone bodies as fuel (Newsholm and Start, 1973; Ruderman *et al.*, 1977). The voles can die after starvation, still having a lot of white and brown fat in their bodies. This is not the case in laboratory rodents (see Newsholm and Start, 1973).

RER in starved *Cl. rufocanus* is a little lower than in *Cl. rutilus*. The reason for this is unclear. The quantity of oxygen consumed by fed voles at rest is the same as

Table 1. Oxygen consumption in fed and starved voles at rest (minimal) and during exercises (maximal)

	Oxygen consumed (ml/g/hr)			
	Fed		Fasted	
	At rest	During exercise	At rest	During exercise
Cl. rutilus	2.6 ± 0.3 (7)	19.6 ± 1.8 (7)	2.2 ± 0.1 (8)	18.7 ± 1.7 (8)
Cl. rufocanus	2.2 ± 0.3 (6)	17.1 ± 2.1 (6)	2.0 ± 0.2 (6)	16.1 ± 1.9 (6)

Results expressed as mean ± SE, number of animals in parentheses.

observed by other authors (Umantseva, 1980). The rate of increasing of oxygen consumption in fed and starved voles during exercises is similar. The relationship between the oxygen consumption and RER is now under investigation.

CONCLUSION

The data presented here show that glucose production in starved voles is not small, therefore, gluconeogenesis is not weak. However, the oxidation of carbohydrates during this state is rapid, so glucose level at least after 12 hr of starvation falls and the animals die. It is of interest that most voles die after starvation still having a lot of fat. This means that the main fuel used in voles during their starvation is carbohydrates, whereas other starved species oxidize predominantly free fatty acids. The reasons for these differences are not clear.

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THE IMPORTANCE OF THE PERIPHERAL THYROID HORMONE DEIODINATION IN ADAPTATION TO AMBIENT TEMPERATURE IN THE CHICKEN (*GALLUS DOMESTICUS*)

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Abstract—1. Evidence is obtained for a major role of thyroxine to triiodothyronine (and/or reverse-triiodothyronine) conversion in the early phase of cold and warm acclimation.

2. The short term change in deiodination seems to be the first response to changes in environmental temperature, and might precede the thyrotropin release from the pituitary after mild cold exposure.

3. The idea of autoregulation of thyroid hormone metabolism is discussed.

INTRODUCTION

In previous experiments it has been suggested (Rudas and Pethes, 1980) that in the early phase of cold acclimation the peripheral metabolism of thyroid hormones may provide a very rapid response which might be independent from the hypothalamic–hypophyseal (HT–HP) system in chicken. It has been shown that the level of thyroxine (T_4) in the serum decreases rapidly (within 1–2 hr) if chickens are taken from the normal laboratory temperature (23°C) and placed in an environment of 10°C. This unexpected behaviour of the T_4 is proved not to be connected with stress-factors or changes in the haemoconcentration but with the immediate increase of the metabolic clearance rate (MCR) of this hormone. The drop in T_4 concentration is followed by an increase in the serum level of 3,5,3′-triiodothyronine. Since it has been indicated (Klandorf *et al.*, 1978; Kühn and Nouwen, 1978) and then proved directly (Astier and Newcomer, 1978) that thyroxine is converted to triiodothyronine (T_3) in chicken and since it has been found (Rudas and Pethes, 1984) that the rate of this conversion is faster in chickens than in mammals, we suggested, but did not prove, that the periphery might handle thyroid hormones independently from the central, i.e. HT–HP, system. It remains to be answered which part of the peripheral metabolism plays the most important role and whether or not the pituitary is in delay to react upon cold exposure.

The following report gives further evidence of early utilization of the serum T_4 pool, thus strengthening the above-mentioned supposition. It establishes also that the T_4 to T_3, or T_4 to reverse-T_3, conversion is mainly responsible for the rapid disappearance of T_4. By measuring serum thyrotropin (TSH) this paper calls attention to the unresponsiveness of the HT–HP system in the early phase of temperature adaptation of the chicken.

MATERIALS AND METHODS

Experiment 1

Five weeks old cockerels were kept at 23°C for one week. They were then placed in separate temperature controlled chambers (3 groups, 45 animals each). The chickens were provided food and water *ad libitum*. The lighting was constant throughout the experiment (24 hr light). After the one week acclimation period, on the day of the experiment, the temperature in two of the chambers was changed: 10°C was set in one of them and 35°C in the other. One group remained at the original 23°C environment. Sixty minutes after the temperature was set [^{125}-I]-T_4 (185 kBq/100 g bwt; SA: 29.4 GBq/g, Isotope Institute, Hungarian Academy of Science, Budapest) was injected i.v. into 15 cockerels in each group. At 2, 60 and 180 min thereafter blood samples were drawn from five of the animals at each time-point by decapitation. Special attention was paid to the completeness of bleeding. The liver, kidney and the pectoral muscle (*m. pectoralis superficialis*) was then removed, snap-frozen and stored at −20°C for later chromatography of the iodocompounds. From each of the animals plasma samples were taken at 0 and 60 min after setting the temperature. These samples were used to determine the thyroxine, triiodothyronine and reverse-triiodothyronine contents of the plasma before the labelled T_4 was administered (see RIA for T_4, T_3 and r-T_3 later).

Experiment 2

In this experiment the effect of exogenous thyrotropin-releasing hormone (TRH) and TSH on the serum level of T_4 has been investigated. Twenty-five weeks old chickens were divided into two groups and 10 animals were injected each with 10 μg/kg body wt synthetic TRH (Sigma Chem. Co., St Louis) while the other ten animals received 20 μg/kg body wt bovine TSH (Sigma). Both hormones were applied i.v. in 0.9% NaCl in a volume of 2 ml/kg body wt. The injection was followed by serial sampling in the same animal at 30, 60, 90, 120, 150, 180 and 210 min afterwards. The T_4 content of the sera collected were determined by radioimmunoassay (see below).

Experiment 3

Seven groups of five weeks old chickens were acclimated to 23°C and constant light for one week with food and water *ad libitum*. Five animals in each group were utilized. On the day of the experiment the first group was placed in a room at 10°C for 1 hr, the second group was exposed to 4°C for 1 hr, the third to 4°C for 2 hr, and the fourth to −20°C for 1 hr. Three groups (Nos 5–7) remained at the original 23°C temperature. The fifth group was thyroidectomized surgically the day before the experiment and 24 hr later the animals were sacrificed. The sixth group was treated with 1.0 μg/kg body wt

T_3 s.c. per day for five days and was sacrificed on the day of the experiment. The seventh group was injected with TRH (10 μg/kg body wt i.v.) and sampled 10, 30, 60 and 90 min thereafter. The cockerels belonging to group Nos 1–4 were bled after having spent the denoted time interval at the given temperature. The plasma T_4 and TSH concentrations were determined by RIA (see later). Normal values for TSH were assessed from serum pool of twenty individuals kept at 23°C.

Radioimmunoassays

T_4, T_3 and reverse-T_3 serum concentrations were determined by specific RIA (Pethes *et al.*, 1977, 1978; Rudas, 1979). The intraassay variances were 5, 6.8 and 8% for T_4, T_3 and reverse-T_3, respectively. The interassay variance was lower than 9% in the case of T_4 and 10, 9.8 for T_3 and reverse-T_3, respectively. The detection limit of the reverse-T_3 RIA is of interest (see Discussion), being 5 pg/assay tube. There is no specific radioimmunoassay available for serum TSH level in chickens. A heterologous assay was therefore used, which was originally developed for use in humans.*

The intraassay variance of this RIA was lower than 4%. No interassay data are available at present. The applicability of this h-TSH RIA for measuring chicken TSH level was validated in several ways: (a) parallelism of serial dilution of chicken serum with the standard curve was examined; (b) the effect of thyroidectomy on the TSH level was checked; (c) the effect of T_3 treatment on TSH secretion was also investigated; (d) TRH provocation test was used to substantiate the TSH RIA on six cockerels of 23°C adapted animals (10 μg/kg body wt TRH i.v., followed by blood sampling 10, 30, 60 and 90 min thereafter; TSH assayed from pooled sera at each point) (see Exp. 3 (groups 5 and 6) for points b and c above).

The TSH measured is immunoassayable-TSH (i-TSH) and further on it is referred to as i-TSH.

Chromatography

The iodothyronine content and the ratio of the different labelled iodocompounds ([^{125}-I]-T_4, [^{125}-I]-T_3, -reverse-T_3 and ^{125}-I) was determined by carrying out extraction and chromatography of the organs collected in Exp. 1. Tissue extracts were made according to Nejad *et al.* (1975) and the extracts obtained were applied onto thin-layer chromatography following exactly the procedure given by West *et al.* (1965). Quantitative measurement of the individual band was carried out by direct assessment of ^{125}-I activity in each band scraped off the TLC plate.

RESULTS

Experiment 1

The effect of the changes in ambient temperature on the serum level of the thyroid hormones is shown in Table 1. This represents the results of 1 hr exposure to the given temperature. At zero-time (on the seventh day of adaptation to 23°C) there is no difference in serum levels either of T_4 or of T_3 in the groups. The serum level of r-T_3 is undetectable at this point. In accordance with our earlier observations the mild cold exposure (10°C, 1 hr) causes a significant ($P < 0.01$) drop in T_4 level. There is a similar drop in the level of T_4 in the 35°C group. No change was observed in the serum level of T_3 60 min after the alteration of temperature. The reverse-T_3 level, on the other hand, was readily detectable in the warm exposed groups.

*Source: Helene Hospices Civils De Lyon, Lyon, France.

[^{125}-I]-T_4 (i.e. the ^{125}-I activity of its products of degradation) 1 hr after the label was administered is represented in Table 2 (the 60 min results are given only since the values after 180 min were similar to this. At 2 min after administration of the label the activity in the organs were too low to be evaluated). The three main columns represents the three temperatures (10, 23, 35°C) while the subcolumns (T_4, T_3, r-T_3, I) show the proportion of the different ^{125}-I-labelled compounds. The rows in this table indicate the percentage of the total radioactivity found in the particular organ (rows "a"). In rows "b" the proportion of iodocompounds is given, expressed as the percentage of row "a" (latter taken as 100%).

One can conclude that the 2 hr cold exposure (60 min before and 60 min after [^{125}-I]-T_4 application) causes the plasma ^{125}-I activity to drop significantly in the cold-exposed animals (3.7% vs 10.2%, $P < 0.01$). This agrees well with our former observation (Rudas and Pethes, 1980) that the T_4 MCR increases rapidly upon cold effect. The ratio of T_3/T_4 is increased apparently via the enhanced T_4 to T_3 conversion. In the liver one can find only 5.4% of the injected dose if the animals are exposed to cold, while 14.7% of it is found in this organ at 23°C and 10.2% at 35°C. It is important to note that behind the total ^{125}-I activity values, there is a remarkable difference as for the proportion of the iodocompounds in the tissues. In the cold-exposed animals one can find 63% T_3, three times that found at a normal temperature of 23°C. This high proportion of T_3 was also detected in other organs (kidney and muscle). At 35°C the balance of the iodothyronine proportion is shifted towards the production of r-T_3. This is in good accordance with the fact that in warm exposed animals one can find a measurable level of inactive r-T_3 (see Table 1).

Experiment 2

The results which are summarized in Table 3 show clearly that both TRH and TSH were able to raise serum T_4 levels very shortly after i.v. administration (30–90 min).

Experiment 3

The i-TSH and T_4 values obtained in the case of cockerels adapted to 23°C temperature and that from different cold-exposed animals are indicated in Table 4. Parts A and B show the results which serve as indicators for the validity of the i-TSH measurement with heterologous antiserum. The i-TSH demonstrates a three-fold rise after surgical thyroidectomy; on the other hand T_3 treatment for 5 days suppresses i-TSH below the detection limit (0.1 mU/l). The T_4 level is very low in thyroidectomized animals and also in T_3-treated animals.

Part B of Table 4 shows the i-TSH serum levels after TRH provocation test. A four-fold increase was observed 30 min after i.v. administration of TRH.

In the experimental groups (Part C, Table 4) exposed to different degrees and duration of cold, there is no change in the level of i-TSH; however, the T_4 responds with significant decrease ($P < 0.01$) in all of the groups. The most pronounced drop can be observed at −20°C ambient temperature.

Table 1. The effect of environmental temperature on the level of thyroid hormones in 5 weeks old cockerels previously adapted to 23°C

Time	T_4 (nmol/l)			T_3 (nmol/l)			r-T_3 (pmol/l)		
	10°C	23°C	35°C	10°C	23°C	35°C	10°C	23°C	35°C
0 min	15.9	16.4	16.1	2.9	3.0	2.9	—†	—†	—†
	1.2	0.9	0.8	0.1	0.1	0.1			
	(38)	(39)	(40)	(33)	(38)	(38)			
60 min	6.9*	15.4	10.1*	3.1	3.1	3.2			197
	0.7	1.0	0.6	0.1	0.1	0.1	—†	—†	10.2
	(38)	(38)	(39)	(30)	(32)	(38)			(34)

$\bar{X} \pm$SEM (N).
*$P < 0.01$ (0 min vs 60 min).
†Below detection limit.

Table 2. The distribution of ^{125}I total activity and the proportion of iodinated compounds ([^{125}I]-T_4, [^{125}I]-T_3 and [^{125}I]-r-T_3) in different tissues and in the plasma of cockerels exposed to changes of the environmental temperature for 2 hr

Tissue		10°C				23°C				35°C			
		T_4	T_3	r-T_3	I	T_4	T_3	r-T_3	I	T_4	T_3	r-T_3	I
Plasma	a		3.7 ± 0.46(5)*				10.2 ± 1.00(5)				7.2 ± 0.91(5)*		
	b	56	30	—	7	87	3	0.5	7	75	0.9	—	8
Liver	a		5.4 ± 0.8 (5)*				14.7 ± 1.10(5)				10.2 ± 0.82(5)*		
	b	17	63	—	12	18	20	—	51	10	8	11	77
Kidney	a		2.8 ± 0.2 (5)*				6.2 ± 0.5 (5)				3.4 ± 0.71(5)*		
	b	8	72	—	7	6	7	—	75	8	1	15	70
Muscle	a		12.6 ± 1.83(5)				12.2 ± 1.48(5)				9.5 ± 0.94(5)		
	b	4	92	—	3	75	10	—	8	9	3	7	80

a = % of injected dose ± SEM (N); b proportion % if a is considered 100%.
*$P < 0.01$ (vs 23°C).

Table 3. Effect of TRH and TSH administration on the circulating levels of T_4 in 6 weeks old cockerels adapted to 23°C environmental temperature for one week (T_4 nmol/l)

Treatment	Minutes before	Minutes after								Hours after
	1	30	60	90	120	150	180	210	240	8
TRH	16.2	25.9*	20.1*	17.1	16.8	15.9	16.5	17.0	16.1	16.1
(10 μg/kg body wt)	1.48	1.12	1.31	0.92	1.13	0.98	0.48	1.09	1.11	1.31
	(10)	(10)	(10)	(10)	(10)	(10)	(10)	(10)	(10)	(10)
TSH	18.1	24.3	37.2*	45.3*	43.2*	40.2*	59.3*	38.2*	29.2*	15.3
(20 μg/kg body wt)	1.41	1.38	1.81	1.71	2.02	2.20	2.16	2.18	1.95	1.08
	(10)	(10)	(10)	(10)	(9)	(10)	(10)	(8)	(10)	(10)

$\bar{X} \pm$SEM (N).
*$P < 0.01$ (vs 1 min before application of TRH/TSH).

Table 4. Serum T_4 and i-TSH levels in cockerels

	Part A			Part B (TRH treatment i.v. 10 μg/kg body wt)					Part C			
	23°C pool	treatment Tx	T_3	0 min	10 min	30 min	60 min	90 min	10°C 1 hr	4°C 1 hr	4°C 2 hr	−20°C 1 hr
TSH	2.5	7.6*	—	3.7	11.5	13.5	4.4	2.6	2.21	2.11	2.53	2.03
(mU/l)	0.11	0.96							0.62	0.55	0.68	0.11
	(5)	(5)				(6)†			(5)	(5)	(5)	(5)
T_4	18.4	0.5*	1.0*	19.3	21.4	29.3*	32.4*	23.6*	7.5*	8.0*	8.0*	3.6*
(nmol/l)	0.92	0.07	0.3	1.25	1.07	2.02	1.93	0.87	1.14	1.01	1.25	0.75
	(5)	(5)	(5)	(6)	(6)	(6)	(6)	(6)	(5)	(5)	(5)	(5)

Part A and Part B = validation of i-TSH assay, Part C = early phase of adaptation to cold environment. Pool = 20 samples from chickens adapted to 23°C pooled and assayed 5 times; Tx = thyroidectomized; T_3 = triiodothyronine treated; $\bar{X} \pm$SEM (N); *$P < 0.01$ (vs 23°C pool), †pooled sera at each point.

DISCUSSION

The validation of i-TSH assay is of major interest in this work. Scarce data are published on TSH level in birds since no avian TSH preparation is available at present. Beside the fact that mammalian TSH is effective in birds (Pethes and Scanes, 1974) the acceptance of the heterologous RIA applied here may be substantiated by results of ours obtained while validating the assay and by data published recently by other authors using heterologous TSH RIA in birds. Parallelism of serial dilutions of chicken serum with TSH standard, increase of serum i-TSH after thyroidectomy and the disappearance of i-TSH from serum of T_3-treated animals as well as TRH provocation test, strongly suggest that what was measured in this assay might really be interpreted as TSH activity. On the other hand, very similar data were obtained by Almeida and Thomas (1981) who applied heterologous TSH RIA in quails. More supporting evidence from the literature is the study of Bogdan *et al.* (1980), who proved the applicability of human TSH RIA kit in avian species.

In the human and in other mammals like the rat (Mueller *et al.*, 1974; Balsam *et al.*, 1975; Bernal and Escobar del Rey, 1975; Hefco *et al.*, 1975) one can see that cold exposure activates the central (hypothalamic–hypophyseal, HT–HP) axis, which in turn causes the thyroid to produce more T_4 to serve enhanced energy utilization on the periphery. In the chicken there was the same finding (Pitt-Rivers and Tata, 1959) that exposure to lower ambient temperature results in greater secretion rate of thyroid hormones (Mueller and Amezcua, 1959). The early establishment of this primary role of the central HT–HP system in thermoadaptation (Stahl and Turner, 1961; Heindrich and Turner, 1967; McFarland *et al.*, 1966) has been confirmed by more recent results (Assenmacher, 1975).

The role of the peripheral conversion of thyroid hormones in different physiological and pathological states has been reviewed by Cavalieri (1980) and Wartofsky and Burman (1982) with respect to the human. The possibility of conversion of T_4 to T_3 in birds was first demonstrated by Astier and Newcomer (1978). More recently the Belgian group (Kühn and Nouwen, 1978; Decuypere *et al.*, 1980) was first to demonstrate that the peripheral conversion of T_4 to T_3 or to r-T_3 may play an important role in the thermoregulatory process of the domestic fowl. However, they did not examine the early periods following thermal challenge. Other recent studies provided astonishing evidence: Bobek (1980) found that in Japanese quail the T_4 level decreased in the early phase of cold exposure, very similar to what had been described previously (Rudas and Pethes, 1980) and in the present study. However, Bobek's explanation is that the early drop of T_4 level is due to stress effects. Another finding controversial to the classic image is that of Moss and Balnave (1978), who observed a rise of T_4 serum level in chicks exposed to warm ambient temperature.

Present findings strongly suggest that the rapid MCR of T_4 in response to cold exposure (which was observed also by May, 1973, and May *et al.*, 1974, in chickens) is mainly due to an increased T_4 to T_3 conversion which probably precedes any effective response by the central HT–HP system. In other words the otherwise responsive thyroid (Exp. 2) might not be activated by the pituitary during this early acclimation period in chicken (Exp. 3, Table 4, Part C). This finding confirms well the findings published by Fisher and Odell (1971) who could not detect any rise of serum TSH upon short-term cold exposure in the human. Data presented in this paper should be handled with care, however, since the TRH provocation test showed that a TSH surge may occur within an hour after TRH application (Table 4, Part B). TSH treatment, on the other hand, causes T_4 level to rise significantly ($P < 0.01$) 1 hr after TSH application, and this effect lasts for several hours. The above findings suggest that even if TSH surge upon cold exposure had been overlooked in Exp. 3 (i.e. before 1 hr of cold exposure, Table 4, Part C) this ought to have led to a long term (1–4 hr) rise in T_4 level. On the contrary, we found that T_4 level dropped intensively upon cold exposure and at least a major importance of peripheral utilization vs thyroidal secretion may be supposed.

Chromatographic separation of iodocompounds generated after i.v. administration of ^{125}-I-labelled T_4 also confirms the supposition that deiodination is an important step in early thermoadaptation (Table 2). The T_4 to T_3 conversion is enhanced in the liver, kidney and muscle of cold-exposed chickens. Warm environment, on the other hand, results in r-T_3 generation. (It should be noted that not all values in rows "b" of Table 2 add up to 100%. This fact refers to the possible importance of formation of small amounts of iodocompounds not identified in this TLC system.)

There are conflicting data on the existence of r-T_3 in the blood of chickens. Premachandra *et al.* (1977) found that serum r-T_3 concentration is close to the detection limit. Decuypere *et al.* (1980) could not detect any r-T_3 in chickens at normal laboratory temperature, neither could we in this or earlier papers (Rudas and Pethes, 1980). Thommes and Hylka (1978) found detectable r-T_3 in chickens, but only at very young ages. The present finding that r-T_3 appears in the serum after warm exposure and that it can be detected in the form of ^{125}-I-r-T_3 in tissues shows that 5-deiodination is also a physiological pathway of T_4 degradation in the bird. One can suppose that the degradation of r-T_3 is too rapid at normal ambient temperatures and this does not allow its determination from serum by RIA. (Extraction and/or concentration of normal serum could be a solution to detect low amounts of r-T_3 in normal serum. This procedure was not introduced, since artifact formation may result in misleading data in the case of non-direct radioimmunoassays of thyroid hormones, Fisher and Dussault, 1971).

On the basis of the results we suggest that cold and warm adaptation of the thyroid economy is a two-step process in birds. First the activation (T_3 formation) or inactivation (r-T_3 formation) of the secreted T_4 will be enhanced according to cold or heat exposure, respectively. Later on the central (HT–HP) system will probably be mobilized to meet the requirements of long-term exposure to changes in ambient temperature.

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THE IMPORTANCE OF EXTERNAL MAGNESIUM IN THE SENSITIVITY OF THE SODIUM EFFLUX TO INJECTED CYCLIC AMP: THE BARNACLE MUSCLE FIBRE AS A PREPARATION

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Abstract—1. The dependence of the response of the Na efflux in ouabain-poisoned barnacle muscle fibres on the external Mg^{2+} concentration has been investigated.

2. External Mg^{2+} suppresses stimulation by injected cAMP of the ouabain-insensitive Na efflux over a wide concentration range. The response of fibres suspended in 50 mM Mg^{2+}-ASW is rather small.

3. By contrast, the response of fibres suspended in 50 mM Mg^{2+}-ASW containing 1-propyl-3-methyl-7-(5-hydroxy-hexyl)-xanthine to the injection of cAMP is quite large.

4. The response of fibres suspended in 50 mM Mg^{2+}-ASW to the injection of catalytic subunit of cAMP–protein kinase is also large.

5. These results give support to the view that a persistent catalytic reaction is a condition which reduces the suppressive action of external Mg^{2+}.

INTRODUCTION

It is typical of barnacle muscle fibres to respond to the injection of cAMP by showing a transitory rise in the Na efflux. Decay of the response can be prevented by applying the xanthine derivative, 1-propyl-3-methyl-7-(5-hydroxyhexyl)-xanthine (PMX) (Nwoga and Bittar, 1983). Behaviour of this type, namely sustained stimulation of the ouabain-insensitive Na efflux, is also seen following the injection of catalytic subunit of cAMP–protein kinase (Bittar *et al.*, 1982). In these two situations, the response of the Na efflux is readily abolished by pre- or post-injecting Mg^{2+} in high concentration. Such results fit together and provide evidence for the view that a low pMg is inhibitory to the catalytic reaction (Granot *et al.*, 1979; Stralfors and Belfrage, 1982). The question now is: Does a raised external Mg^{2+} affect the sensitivity of the Na efflux to injected cAMP? The purpose of this communication is two-fold: *first*, to describe experiments which show that a raised external Mg^{2+} concentration suppresses the response of the ouabain-insensitive Na efflux to the injection of cAMP and that this is overcome by preapplying externally the xanthine derivative, PMX. And *second*, to draw the attention to experimenters using ASW containing 50 mM Mg^{2+} that studies of cAMP action on Na movements might not be fruitful unless the Mg^{2+} concentration is reduced quite markedly.

MATERIALS AND METHODS

Specimens of the barnacle *Balanus nubilus* were supplied by Mr David King of Friday Harbor, Seattle, Washington. The experiments were carried out in the same way as those described previously (e.g. Nwoga and Bittar, 1983). Solutions of artificial sea water (ASW) with different Mg^{2+} concentrations were prepared by lowering (or raising) NaCl in osmotically equivalent amounts. The sample of catalytic subunit of cyclic AMP-dependent protein kinase type 2 was a gift from Professor E. Fischer of the Department of Biochemistry, University of Washington, Seattle, Washington. 1-Propyl-3-methyl-7-(5-hydroxy-hexyl)-xanthine was a gift from Dr V. Stefanovich, Hoechst Aktiengesellschaft, 6200 Wiesbaden 12, Federal Republic of Germany.

RESULTS

In the first group of experiments, 10^{-3}M cAMP was injected into fibres suspended in 10^{-4}M ouabain, 50 mM Mg^{2+}-ASW and into fibres suspended in 10^{-4}M ouabain, 10 mM Mg^{2+}-ASW. The results obtained show that the response to cAMP injection is markedly reduced by elevating the external Mg^{2+} to 50 mM [89 ± 33% (SEM) stimulation, $N = 4$ vs 1028 ± 238% stimulation, $N = 4$, $P < 0.01$]. In view of this finding, more experiments of this type were done over a range of external Mg^{2+} concentration. The results of these experiments are summarized in Fig. 1, where it can be seen that as the external Mg^{2+} concentration is

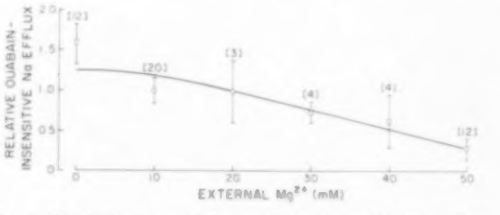


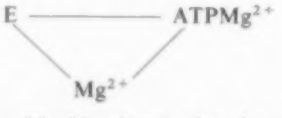
Fig. 1. The behaviour of the ouabain-insensitive Na efflux towards the injection of 10^{-3}M cAMP with variation of the external Mg^{2+} concentration. Ordinate is the maximal rate constant for the ouabain-insensitive Na efflux observed following cAMP injection relative to that measured in ASW containing 10 mM Mg^{2+}. Vertical bars represent ± SE of the mean. The number of observations involved is recorded in parentheses.

reduced (from 50 mM), there is an increase in the sensitivity of the Na efflux to the injection of 10^{-3}M cAMP. This tends to level off in the 10–20 mM Mg^{2+} range. The difference between the response obtained with 10 mM Mg^{2+}-ASW and that with nominally Mg^{2+}-free ASW is not significant (based on experiments involving fibres isolated from the same barnacle specimens: $587 \pm 123\%$, $N = 8$ vs $855 \pm 164\%$, $N = 8$, respectively, $P < 0.3$). It is also noteworthy that in eight experiments using 50 mM Mg^{2+}-ASW, the external Mg^{2+} concentration was reduced to 10 mM following the onset of peak stimulation by injected cAMP. However, a return to 10 mM Mg^{2+} failed to stop the response to cAMP from decaying.

The protein kinase theory states that cAMP promotes the dissociation of R from the catalytic subunit to produce the active form of the enzyme and that free C (the active kinase) elicits the physiological response by phosphorylating specific cellular proteins (Greengard, 1978; Flockhart and Corbin, 1982). Thus, the question arising is whether a raised external Mg^{2+} concentration, e.g. 50 mM, reduces the response of ouabain-poisoned fibres to injected catalytic subunit. To answer this, 5×10^{-5}M catalytic subunit (in a 3 mM HEPES solution, pH 7.2) was injected into fibres suspended in 10^{-4}M ouabain, 50 mM Mg^{2+}-ASW and control fibres suspended in 10^{-4}M ouabain, 10 mM Mg^{2+}-ASW. The results show $444 \pm 84\%$ stimulation, $N = 11$ vs $579 \pm 93\%$ stimulation, $N = 12$, respectively, P being < 0.4. This unexpected result prompted us to carry out experiments with the cAMP-phosphodiesterase inhibitor, 1-propyl-3-methyl-7-(5-hydroxy-hexyl)-xanthine (PMX), the rationale being that the application of PMX followed by the injection of cAMP results in persistence of the catalytic reaction (Nwoga and Bittar, 1983; Bittar and Chambers, unpublished). Expressed in another way, the kinetic results obtained by using PMX bear a striking resemblance to those seen following the injection of catalytic subunit. The results of these experiments indicate that ouabain-poisoned fibres suspended in 50 mM Mg^{2+}-ASW containing 10^{-3}M PMX show $1669 \pm 426\%$ stimulation ($N = 8$) following the injection of 10^{-3}M cAMP. This value is significantly greater than the $264 \pm 121\%$ stimulation ($N = 6$) found in ouabain-poisoned fibres suspended in 50 mM Mg^{2+}-ASW (without PMX), following injection of 10^{-3}M cAMP, P being < 0.02.

DISCUSSION

Granot *et al.* (1979) and Armstrong *et al.* (1979), who studied the interaction of ATPMg with the catalytic subunit of cAMP–protein kinase by nuclear magnetic resonance, have put forward the view that excess Mg^{2+} is strongly inhibitory to kinase activity. Suppression is thought to be due to bridge formation between the enzyme (E) and ATPMg. That is,



Compatible with this view is the observation that Mg^{2+} injection into barnacle fibres reduces the response of the Na efflux to injected cAMP or catalytic subunit. The fact that raising the external Mg^{2+} from 10 to 50 mM markedly reduces the response to injected cAMP but fails to do so in the case of fibres pretreated with PMX, rules out the possibility that myoplasmic pMg in fibres bathed in 50 mM Mg^{2+}-ASW falls to a level which is sufficiently inhibitory to the catalytic reaction. As will be recalled, the internal free Mg^{2+} concentration in barnacle fibres is about 6 mM (Scarpa and Brinley, 1981). However, this concentration of Mg^{2+} is not high enough to inhibit the catalytic reaction (Reimann *et al.*, 1971; Chiu and Tao, 1978). For inhibition to occur, a concentration in excess of 10 mM (Stralfors and Belfrage, 1982) or 20 mM (Armstrong *et al.*, 1979) is required. Moreover, measurements made with Mg^{2+}-selective microelectrodes indicate that a raised external Mg^{2+} leads to a rather slow and small rise in internal Mg^{2+} activity, e.g. in frog skeletal muscle (Hess *et al.*, 1982; Lopez *et al.*, 1983). Assuming that this is also so in barnacle fibres, it may be deduced that the fall in myoplasmic pMg is insufficient to inhibit the catalytic reaction in fibres suspended in 50 mM Mg^{2+}-ASW and injected with catalytic subunit, as well as in fibres suspended in 50 mM Mg^{2+}-ASW containing PMX and injected with cAMP.

In seeking an interpretation of the present results, the role of the catalytic reaction seems critical. It is plain that when the catalytic reaction is transitory, the response to cAMP is suppressed by high external Mg^{2+}, and when the reaction is persistent, the effect of high external Mg^{2+} is much reduced. In other words, the degree of competition between external Mg^{2+} and Ca^{2+} for exchange with internal Na is determined by membrane phosphorylation.

Finally, it is consistent with such data to admit that the physiological significance of cAMP as a modulator of Na efflux in barnacle fibres remains unclear. This state of affairs is entirely due to the absence of information concerning the *activity* of Mg^{2+} in barnacle plasma and in ASW. It suffices to say that the experiment where the response of the Na efflux to injected cAMP is studied after rendering the Mg^{2+} activity in ASW roughly equal to that found in barnacle plasma needs to be done.

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ON THE MEMBRANE POTENTIAL INDEPENDENT MECHANISM OF THE SODIUM PUMP-INDUCED INHIBITION OF SPONTANEOUS ELECTRICAL ACTIVITY OF JAPANESE LAND SNAIL NEURONS

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(*Received* 4 *May* 1983)

Abstract—1. The dependence of excitability on pump activity of bursting pacemaker of Japanese land snail neurons was studied.

2. The incubation of neurons in potassium-free solution, in spite of the fact that the membrane was hyperpolarized, the frequency of bursting activity was increased and sometimes the interburst hyperpolarization fully disappeared.

3. The 4 mM potassium or rubidium pump activation after preincubation of the neurons in potassium-free solution leads to transient inhibition of bursting activity independent of the membrane potential level.

4. Adrenaline has a potentiation effect on the potassium-induced inhibition of electrical activity after preincubation of the neurons in the potassium free solutions. This effect of adrenaline was absent in ouabain containing medium.

5. Increasing the external medium tonicity has an inhibitory effect on electrical activity of neurons.

6. It was suggested that the pump-induced cell volume changes could be one of the membrane potential independent mechanisms by which Na–K pump could modulate the membrane excitability.

INTRODUCTION

It is well-known that the electrogenic Na-pump can modulate the electrical activity of cells by hyperpolarization of the membrane, which leads to the increasing of threshold of the action potential. There are many reviews on this problem in literature (Kerkut and York, 1971; Koketsu, 1971; Thomas, 1972; Ayrapetyan, 1974). It was suggested that by the above-mentioned mechanism the electrogenic Na-pump can regulate the synaptic activity (Nishi and Koketsu, 1968; Kerkut *et al.*, 1969) and spontaneous pacemaker activity (Ayrapetyan, 1969, 1977; Carpenter and Alving, 1968; Carpenter, 1973; Remey and Arvantaki Chalazonitis, 1970).

In recent experiments on snail neurons (Ayrapetyan and Sulemanian, 1979) and on the slices of mammalian brain (Cook, 1978) it was shown that the activation and inactivation of Na-pump are accompanying by outflow and inflow of water through the membrane and it was suggested that this mechanism can be an important mechanism by which metabolism regulates the cell volume.

The problem of physiological significance of the pump-induced cell volume changes in the regulation of electrogenic and chemogenic properties of the membrane was the subject of recent investigations of Ayrapetyan *et al.* (see review Ayrapetyan, 1980). It was shown that the inactivation of Na-pump leads to an increase, while its activation leads to a decrease of the acetylcholine sensitivity of snail neurons by, correspondingly, changes of the number of acetylcholine receptors in the membrane (Ayrapetyan and Arvanov, 1977, 1979; Ayrapetyan, 1980).

On the other hand in experiments on bullfrog's sympathetic ganglion neurons and on the muscle fibers by Koketsu *et al.* (1982a, b) it was shown that the adrenaline has a strong modulatory effect on the chemogenic and electrogenic properties of the membrane by an unknown mechanism.

Investigations of the adrenaline effect on ^{22}Na efflux, a strong activation effect on the Na-pump activity was shown. Therefore, two questions are proposed:

(1) Do the pump-induced volume changes have an effect on the membrane excitability?

(2) Do the pump-induced volume changes underly the modulation effects of adrenaline on the membrane electrical activity?

As an experimental model the pacemaker activity of Japanese land snail neurons was chosen.

MATERIALS AND METHODS

The whole circumoesophagel ganglion of *Euhadra congenita hickonis* Kobelt was isolated. Experiments were carried out on the neurons. These neurons were exposed by using fine forceps and razor to dissect away the connective tissue above the cell.

Microelectrodes used for recording were filled with 3 M KCl. The resistance of electrodes was 10–20 MΩ.

A 3 M KCl–agar-filled glass bridge served as the reference electrode. The recording electrode and reference electrode were connected to a negative capacitance amplifier with high-input impedance, displayed on an oscilloscope and recorded by a pen recorder.

The normal snail Ringer used had the following composition: Na isethionate 80 mM, KCl 4 mM, $CaCl_2$ 7 mM, $MgCl_2$ 5 mM, $NaHCO_3$ 5 mM. The reason why we use sodium isothionate instead of sodium chrloride in normal Ringer solution was to eliminate the shunting effect on

sodium pump electrogenicity (Ayrapetyan, 1974). Potassium-free solution was made by adding 60 mM sucrose into external bathing solution.

Ouabain (10^{-4}M), adrenaline (10^{-4}M) and acetylcholine (10^{-4}M) were made up freshly in Ringer for each experiment.

RESULTS

External potassium effect on spontaneous activity

After being penetrated by a microelectrode the pacemaker neurons usually fire steadily at relatively high rates for 2–5 min, after which they assume the pattern of rhythmic or regular bursting pacemakers or become silent. The silent pacemaker neurons become active when the membrane was depolarized by applying outward current, by increasing external potassium concentration or by transferring to K-free solution.

Figure 1(A) illustrates the electrical activity of parabolic bursting neuron in normal Ringer solution containing 4 mM K after 5 min penetration. This neuron is in the same position in the right parietal ganglion as the RPa1 neuron in *Helix* (Sakharov and Salanki, 1969). After 5 min penetration by microelectrodes this neuron becomes silent and has a membrane potential of (MP) = 60 mV.

Removing potassium ions from the external medium has a biphasic effect on the MP: during the initial 3 min the membrane was hyperpolarized until 64 mV and then gradually depolarized to the 53 mV after 13 min in K-free solution when bursting pacemaker activity again was appeared (Fig. 1B). As it can be seen in this figure, in spite of that level of MP following incubation in K-free solution was not significantly changed, the duration of burst increased gradually and finally the interburst hyperpolarization disappeared and this neurone, which usually has parabolic bursting activity in normal Ringer solution, beats. From these data it can be suggested that during incubation of neuron in K-free solution, though the MP level was not significantly changed, its membrane excitability was increased.

It was shown that the K-free induced transient hyperpolarization is caused by increase of the electrogenicity of Na-pump because of the decreased coupling between sodium efflux and potassium influx (Kostuk *et al.*, 1972; Ayrapetyan, 1976). This hyper-

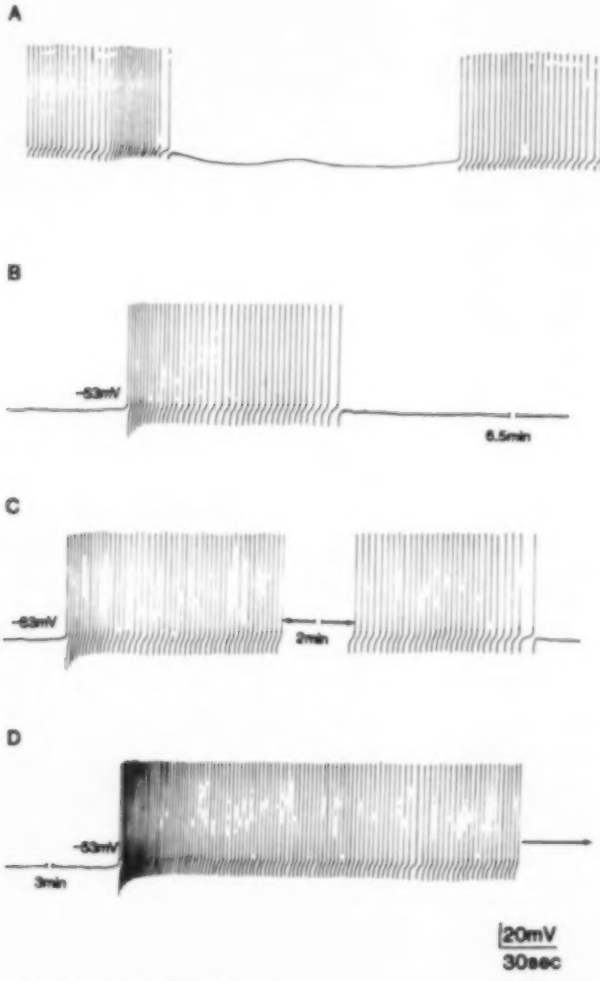

Fig. 1. Electrical activity of parabolic bursting neuron in normal Ringer solution containing 4 mM K (A) and in K-free solution after 19 min (B), 29 min (C) and 40 min (D) incubation.

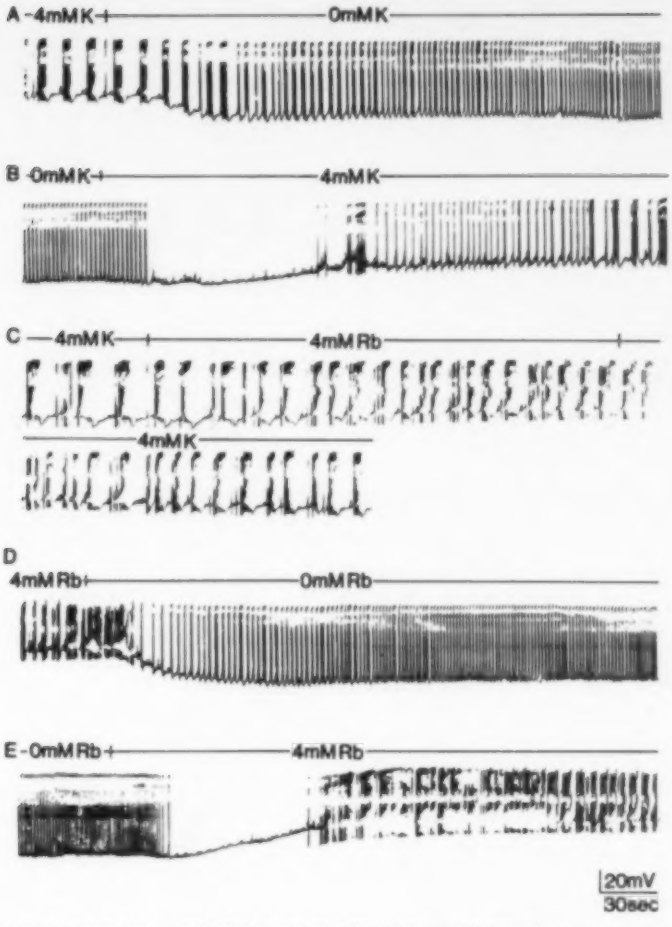

Fig. 2. The effect of 0 mM K (A), 4 mM K (B), 0 mM Rb (D) and 4 mM Rb (E) induced hyperpolarizations and 4 mM K replacement by 4 mM Rb (C) in external solution on bursting activity of pacemaker neurons.

polarization depends on activity of Na-pump in the K-free solution, and was inhibited by ouabain. Therefore the K-free solution can produce membrane hyperpolarization by two ways: (1) by increasing the diffusion part of MP; (2) by increasing the metabolic pump component of MP.

In previous experiments (Ayrapetyan, 1969b) it was shown that high external potassium-induced hyperpolarization in snail neurons was caused by activation of the electrogenic Na-pump.

Thus, there are two possibilities to produce the increase of pump dependent part of MP: (1) by applying K-free solution, which leads to increase of Na-pump electrogenicity, (in spite of depressing net sodium by decreasing coupling ratio between sodium efflux and potassium influx); (2) by potassium induced stimulation of sodium efflux. Both K-free induced and potassium induced membrane hyperpolarization are transient and later after prolonged incubation of neurons in corresponding solution the properties of the membrane become close to the potassium electrode values (Ayrapetyan, 1976).

In order to find out, whether the Na-pump activity itself modulates the electrical activity of the membrane excitability independently its electrogenic effects, 0 and 4 mM K-induced transient hyperpolarization on the pacemaker activity were studied. Figure 2 shows the effect of 0 mM K-induced (A) and 4 mM K-induced hyperpolarization (B) on the frequency of bursting activity in the same neuron. 0 mM K-induced hyperpolarization of the membrane was accompanied by a marked increase of bursting activity of the neuron, while 4 mM K-induced hyperpolarization had an opposite effect on the frequency of bursting activity. In the spontaneous activity the neuron was completely depressed. The inhibition of electrical activity by 4 mM K-induced activation of the pump does not significantly change the level of MP. The MP-independent Na-pump inhibition of impulse activity appeared more clearly when in normal Ringer solution the potassium ions were replaced by rubidium ions. Potassium ions can be effectively replaced by rubidium ions to study Na-K-pump as rubidium ions are taken up by the potassium uptake mechanism (Adrian and Slayman, 1966).

As shown in Fig. 2(C), 4 mM K replacement by 4 mM Rb leads to 4–6 mV depolarization of MP, which was accompanied by increased bursting activity of the neuron. Removal of rubidium ions from the external solutions (Fig. 2D) had the same effect on electrical and MP as in removal of potassium ions (Fig. 2A). After 7 min preincubation of the neuron in Rb- and K-free

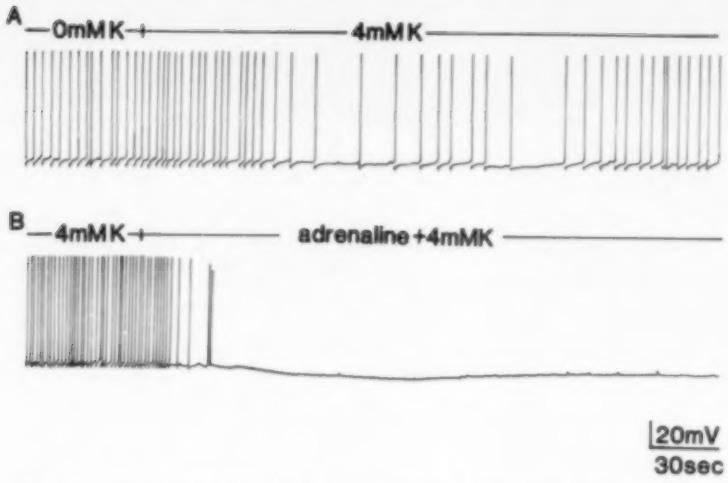

Fig. 3. The effect of adrenaline on the 4 mM K-induced hyperpolarization of membrane after the preliminary incubation of neurons in K-free solution during 7 min. Four mM K-induced hyperpolarization in normal Ringer solution (A), 4 mM K-induced hyperpolarization in the presence of 10^{-4} M adrenaline in external solution.

solution the 4 mM Rb-induced activation of Na-pump depresses its spontaneous activity (Fig. 2E). During the 4 mM Rb-induced inhibition of electrical activity on the level of MP which was lower (−20 mV) than its proceeding level in K-free solution, the impulse activity of neurons remained in the inhibition state.

These data suggest that Na-pump activation leads to inhibition of impulse activity not only by increasing MP level but also by an MP-independent mechanism.

Adrenaline effect on the spontaneous activity

In order to find out whether the adrenaline inhibition on the electrical activity (Minota and Koketsu, 1977; Koketsu and Akasu, 1982; Koketsu *et al.*, 1982) of a neuron is due to its activation of the Na-pump (Kaibara *et al.*, 1982) the adrenaline effect on the pacemaker activity of neurons was studied in Na-pump active and inactive states. Figure 3 shows that adrenaline leads to significant increase of the both amplitudes and the duration of 4 mM K-induced hyperpolarization after the 7 min pre-incubation the neuron in K-free solution. These results agree with previous experiments on the muscles (Kaibara *et al.*, 1981) on the activation effect of adrenaline on the ^{22}Na efflux. As it can be seen in Fig. 3(C) adrenaline, as in the case of sympathetic ganglia of bullfrogs (Minota and Koketsu, 1977), depresses amplitudes of the both action potential and its after hyperpolarization. The

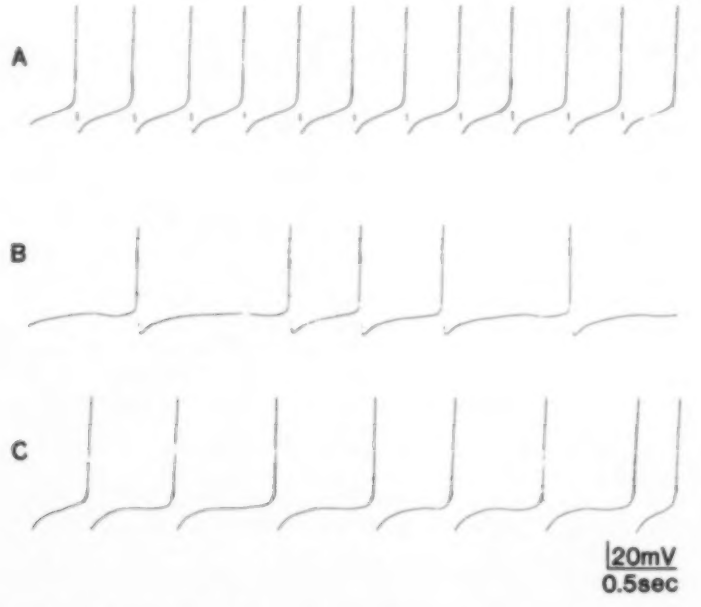

Fig. 4. The adrenaline effect on frequency of spontaneous activity of neuron in normal (B) and K-free solution (C). (A) is the control spontaneous activity in normal Ringer solution.

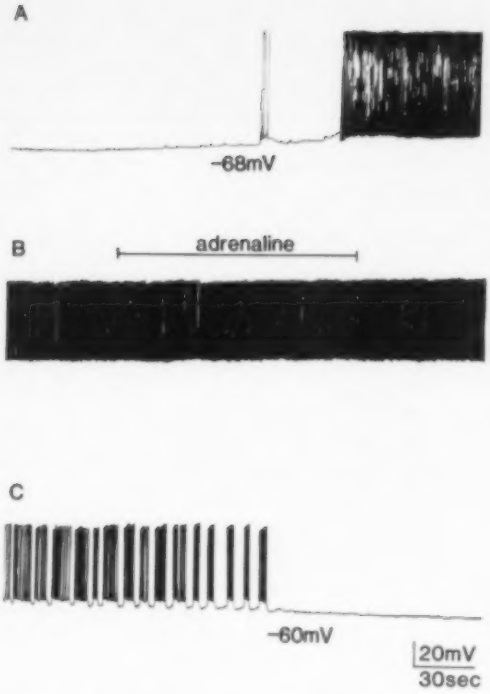

Fig. 5. The disappearance of inhibitory effect of adrenaline on spontaneous activity in ouabain containing solution. The depolarizing effect of ouabain in normal Ringer solution (A), the effect of adrenaline on frequency of spontaneous activity (B), the restoration of membrane potential to initial level after long incubation (C).

inhibitory effect of adrenaline is due not only to adrenaline induced hyperpolarization but also to a direct effect on the generation of the action potential. The inhibitory effect of adrenalin on the pacemaker activity can be demonstrated on neurons which have high frequency responses and (because of low input resistance) which did not produce an electrogenic effect of the adrenalin-induced activation of Na-pump (Fig. 4). As illustrated in this figure, in spite of decreasing the level of MP in the adrenalin medium the frequency of discharges was significantly decreased. In the same neuron such inhibitory effect of adrenaline medium was disappeared in K-free solution. After some time in K-free solution the opposite effect can produce, i.e. increasing frequency of spontaneous activity (Fig. 4C).

The inhibitory effect of adrenalin was also absent in ouabain medium (Fig. 5). This data indicates that adrenalin-induced inhibition of impulse activity is realized by activation of Na-pump activity and does not depend on MP level.

Sucrose effect

The activation of the Na-pump is accompanied by water flow from inside to outside of the membrane, which leads to shrinking of the cell (Ayrapetyan and Sulemanian, 1979). In order to find out whether the pump-induced volume changes underly the MP-independent mechanism of inhibition of pacemaker activity, we studied the effect of hypertonicity of the external medium on the pacemaker activity. In hypertonic solution the snail giant neurons after transferring in hypertonic medium immediately shrink by losing water (Ayrapetyan, 1980).

Figure 6 shows the effect of increasing tonicity of the external bathing solution on the neuron which has parabolic bursting activity. This neuron in normal Ringer solution was silent and after applying 10 mM K the membrane was depolarized in 2 min by 10 mV and generated spontaneous activity (Figs 6A and C). After transferring the neurons to hypertonic solution these neurons become active in 10 mM K only after 6 min. In hypertonic solution the frequency of impulse activity is significantly decreased, i.e. we have the same picture

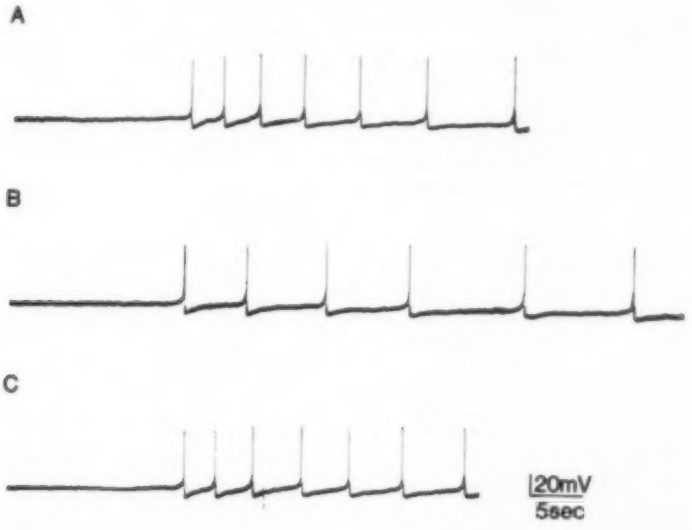

Fig. 6. The depressing effect of hypertonic solution on frequency of impulse activity during the 10 mM K-induced burst of firing in the parabolic bursting neuron. The beginning of burst in isotonic 10 mM K Ringer solution before (A), after (C) and in hypertonic (containing 60 mM sucrose) 10 mM K Ringer solution (B).

as in the case of adrenalin-induced pump activation (Fig. 4).

DISCUSSION

From the classical membrane theory, suggesting that the physiological role of the Na–K-pump is limited to its restoration of ionic gradients across the membrane after generation of action potential during which there are increased sodium and decreased potassium inside the membrane. According to this theory, the Na–K-pump can modulate excitability of the membrane only by changing the ionic gradients on the membrane (Hodgkin, 1964). The electrogenic character of the Na–K-pump has been demonstrated in all excitable cells (see book by Kerkut and York, 1971; reviews by Thomas, 1972; Koketsu, 1971; Ayrapetyan, 1974) in the case of squid axon also (De Weer and Gedulding, 1973). Therefore the second mechanism, by which the Na–K-pump can modulate excitability of the membrane is by the pump-induced membrane potential change. It was shown that the pump-induced MP change can be modulated by synaptic activity (Nishi and Koketsu, 1968; Koketsu, 1971; Kerkut *et al.*, 1969) and spontaneous activity of nerve cells (Carpenter and Alving, 1968; Carpenter, 1973; Ayrapetyan, 1969, 1976; Romey and Arvantaki-Chalazonitis, 1970).

The idea of the electrogenic character of the Na–K-pump proposes that the chloride and sodium extruded is in excess of absorbed potassium, so that the operation of the Na–K-pump would effect a net loss of cellular salt together with osmotically associated water and pump activation or inhibition should result in cellular shrinking or swelling. On the isolated snail giant neurons it was demonstrated that the pump regulates the cell volume (Ayrapetyan and Sulemanian, 1979). Such regulation of cell volume has a physiological significance in the regulation of number of acetylcholine receptors (Ayrapetyan and Arvanov, 1979; Ayrapetyan, 1980), and pump units (Ayrapetyan, 1980) on the membrane and the membrane permeability (Sulemanian, 1980).

The present results, that 0 mM K-induced Na-pump inactivation, when the level of MP significantly does not change (Fig. 1) or the membrane was hyperpolarized (Fig. 2), leads to stimulation of spontaneous activity while 4 mM K, 4 mM Rb (Fig. 2) and adrenalin (Figs 3 and 4)-induced Na-pump activation independent of the level of MP leads to inhibition of spontaneous activity, strongly suggests the presence of MP-independent Na–K-pump inhibition mechanism on the spontaneous activity of the cell. The disappearance of the inhibition effect of adrenalin on the pacemaker activity in K-free and in ouabain-containing medium, indicates that the modulation effect of adrenalin on the electrical activity of the neurons is due to induction of the Na–K-pump, MP-independent inhibition of spontaneous activity of the cell. The cell swelling—induced by inhibition of the Na–K-pump or by hypotonic solution—is accompanied by increased membrane permeability (Sulemanian, 1980) and membrane chemosensitivity (Ayrapetyan and Arvanov, 1979). The increased membrane excitability in K-free solution (Figs 1 and 2) may be explained by 0 mM K-induced swelling of the cell. The data that the hypertonic solution has the same effect on the frequency of spontaneous activity (Fig. 6) as 4 mM K or Rb and adrenaline induced Na–K-pump activation (Fig. 2) suggested that the pump-induced cell volume changing could be one of the MP-independent mechanism by which the Na–K-pump could modulate the membrane excitability.

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ADAPTATION OF GREEN TOAD (*BUFO VIRIDIS*) TO TERRESTRIAL LIFE BY UREA ACCUMULATION

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Abstract—1. The green toad (*Bufo viridis*) reaches in Israel the southern limit of its distribution and penetrates arid zones. Throughout the dry season (6–8 months) it stays out of the water and uses protective terrestrial shelters.

2. In the present study green toads were tested in the laboratory following their prolonged exposure to terrestrial conditions in drying soil.

3. After three months in the soil blood plasma concentration of the toads reached a maximum of 1400 mOsm/kg with an urea concentration of 900 mM. Intracellular urea concentration in the muscles of these toads amounted to only 500 mM.

4. Linear correlations were demonstrated between plasma osmolality and plasma urea concentration and between plasma urea concentration and urea concentration in the muscle.

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INTRODUCTION

The green toad (*Bufo viridis*) is a terrestrial anuran living in a wide range of habitats, including most arid regions (Warburg, 1971, 1972). In Israel it reaches the southern limit of its distribution and penetrates even desert. Throughout the long (6–8 months) and dry summer the green toads in Israel stay out of water in protective soil shelters with no access to free water. The capacity of the green toad to cope with osmotic stress has been studied so far in the laboratory by exposing the animals to saline solution of increasing concentrations (Gordon, 1962; Katz, 1973). Katz succeeded in adapting these toads to concentrations as high as 800 mOsm/kg NaCl. Saline aquatic media, however, are not relevant to the natural habitat of the terrestrial green toad.

In the present study we focused on the way toads cope with the terrestrial environment. Similar studies have been carried out so far in a few American anurans (McClanahan, 1972; Shoemaker, 1964; Shoemaker *et al.*, 1969). The spade-foot toad (*Scaphiopus couchii*), similarly to the green toad, spends prolonged periods of time in protective terrestrial shelters and an elevated osmolality of the body fluid was reported. It was suggested that presumably such a characteristic enables the toad to absorb soil moisture and survive the dry summer. Urea was found to contribute significantly to the high osmotic concentration in these toads. In the present study green toads were maintained in the laboratory in dry soil under conditions similar to their summer shelters. The osmolality of their body fluid, plasma electrolytes and intracellular urea concentrations were assessed following a prolonged exposure to dehydrating conditions in the soil.

MATERIALS AND METHODS

Green toads (*Bufo viridis*) were collected during the winter months from the Mediterranean coastal plain of Israel and were kept in the laboratory in terrarias containing sandy soil. Water was added to the soil twice a week to full field capacity, at a temperature of 20–25°C.

In the summer, water was no longer added and so the soil gradually dried up. In addition, water continued to be added to a control of four toads. From the dry soil, three animals were removed monthly during a three month period following dehydration.

Blood samples were taken by heart puncture, using 1 ml syringe, previously washed with lithium heparin. The blood samples were then immediately centrifuged for 10 min at 300 *g*, then treated with TCA (5%) and centrifuged again for 15 min at 300 *g*. Urea concentration was determined in the plasma water (Bruhmann and Hanke, 1980). The urea concentration in muscle samples taken from the hind-leg (200 mg) was determined after homogenization with TCA (5%) and immediate centrifugation for 15 min at 300 *g*. The water content in muscle tissue was determined by desiccating the tissue at 60°C until it reached a constant dry weight. Extracellular space of muscle tissue in the toads was determined by the method of Ling and Walton (1975). The concentration of urea was related to intracellular water content and was calculated by assuming that plasma and interstitial urea concentrations were equal.

Urea was determined colorimetrically by the method of Foster and Hoccholzer (1971). Sodium and potassium in the plasma were determined by atomic absorption (Model 130) and Cl^- by automatic titration (Radiometer chloride-titrator), as described previously by Degani (1981).

RESULTS

In toads that burrow in wet, sandy soil, plasma, osmotic pressure and urea concentrations all increased by increasing the dryness of the sand (Fig. 1).

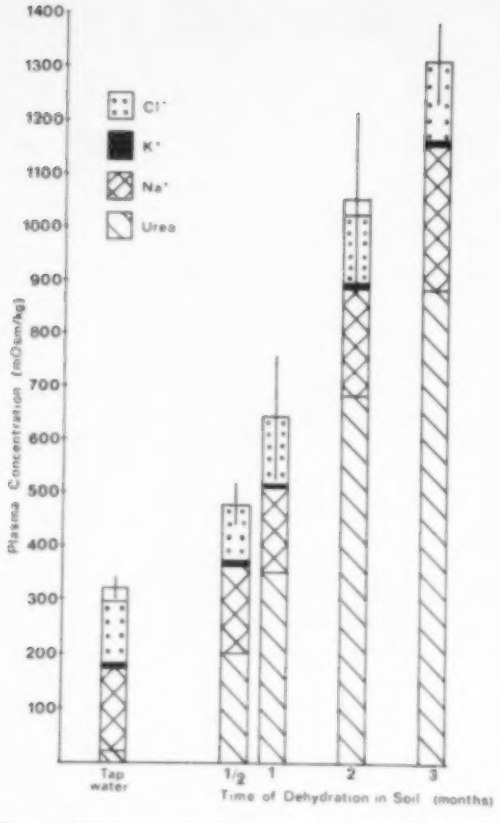

Fig. 1. Plasma concentration and plasma constituents in *Bufo viridis* during progressive dehydration in soil.

After three months the toads were still responsive and agile. Blood plasma concentration amounted to maximum level of 1400 mOsm/kg, the urea concentration being 900 mM (Fig. 1). There is a significant linear relationship between the increased plasma osmotic pressure and urea concentration which can be described by the equation: $y = 0.65p - 122.8$ ($P < 0.01$, $r = 0.97$) where y is plasma urea concentration and p is the plasma osmotic pressure. The rest of the increase in plasma osmotic pressure results from increasing mainly urea concentration and less significantly Na^+ and Cl^- concentration, while accumulation of K^+ is nil (Fig. 1).

There is also a linear relationship between plasma urea concentration and intracellular muscle which can be described by the equations: $y = 0.46 P_r + 44.00$ (where y is intracellular urea concentration and P_r is urea concentration in blood serum). The intercept is significantly positive (Fig. 2). The difference between the concentration of urea in plasma and muscle was less noticeable in low urea concentration than in high urea concentration (Fig. 2).

DISCUSSION

The osmotic concentration recorded in the body fluid of the green toad following a prolonged dehydration in the soil is the highest reported so far for any terrestrial amphibian. Even in the crab-eating frog (*Rana cancrivora*), the only anuran that lives in sea-water, the maximal plasma osmotic concentrations barely exceed 800 mOsm/kg (Table 1). A similar high osmotic concentration was recorded in the terrestrial spade-foot toad (*Scaphiopus couchii*) from the Arizona desert. Values reported for other anurans tested in soil did not exceed that value. Compared to the maximal values reported for the green toads tested in aquatic media of high NaCl concentration, the plasma osmotic concentration in the green toads (*Bufo viridis*) tested in drying soil is remarkably high (Table 1).

Urea concentration contributed significantly to the high plasma osmotic concentrations in all amphibians mentioned above. However compared to a maximal urea concentration of 350 mM in *Rana cancrivore*, 265 mM in *Bufo viridis* tested in 800 mOsm/kg solution, and 584 mM in the burrowing spade-foot toads, urea concentrations of over 900 mM were recorded in the green toads that were maintained in the drying soil. Accumulation of urea is thus conspicuous in all anurans that demonstrate a capacity to elevate significantly their body fluid osmotic concentration, and complements the elevation of electrolytes also recorded in the animal. Nevertheless Na^+ and Cl^- concentrations seem never to exceed significantly 600 mOsm/kg, which is twice the maximal concentration recorded from animals in fresh water. Urea, however, both in spade-foot toads and in the green toad in drying soil, may be 20–30 times more concentrated than in fresh water. Presumably, beyond a certain plasma concentration the accumulation of urea is the main mechanism for achieving high osmolality of body fluid. A terrestrial condition seemed to favour the elevation of plasma osmotic concentration and the accumulation of urea far better than aquatic media of high salinity.

The accumulation of urea affects not only the extracellular (EC) fluid. Following the exposure of the green toads to dehydrating conditions in the soil, intracellular urea was also elevated. Unlike the intracellular urea concentration in green toads from fresh water that is similar to or exceeds that of the plasma urea concentration, the intracellular (IC) urea in the animals from the soil is less concentrated than in the plasma and never exceeded 500 mM. Such an uneven

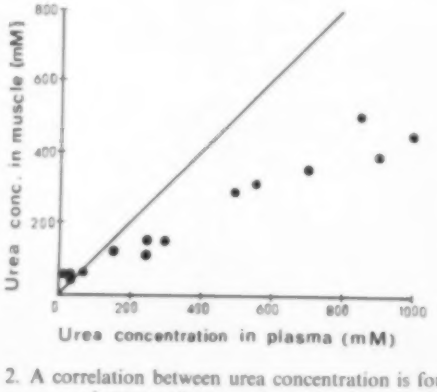

Fig. 2. A correlation between urea concentration is found between plasma and muscle.

Table 1. The plasma osmotic concentration and composition in selected species of Amphibia

Species	Concentration (mOsm/kg)	Na^+ (mM)	Cl^- (mM)	Urea (mM)	References
		Saline adaptation			
Rana cancrivora					
Tap water	290	125	98	40	Gordon *et al.* (1961)
80% S.W.	830	252	227	350	
Bufo viridis					
Tap water	270	113	99	38	Katz (1973)
770 mOsm/kg	805	285	240	265	
		Terrestrial adaptation			
Scaphiopus couchii					
Tap water	305	159		39	McClanahan (1967)
Emerging in summer (from aestivation)	630	280		228	
4 months in soil	235	208		584	McClanahan (1972)
Ambystoma tigrinum					
9 months in soil	550			220	Delson and Whitford (1973)
Salamandra salamandra					
Tap water	239	100	82	17	Degani (1981)
1.5 months in soil	636	267	200	223	
Bufo viridis					
Tap water	330	120	100	20	This study
2 months in soil	983	198	140	627	
3 months in soil	1320	231	181	900	

distribution of urea may be related to the high protein and metabolic concentration in the IC than in the EC compartment. Whether this characteristic helps the toad's cells withstand the high plasma urea concentration was not clarified in the present study.

In toads collected in the field, during the summer osmotic concentration did not exceed 800 mOsm/kg. Plasma and urea in these animals were concentrated to only 300 mM (Degani *et al.*, 1981). But these animals, however, seemed fully hydrated with bloated urine bladders. Presumably their ability to increase their osmotic concentration, and as a result also their capacity to absorb water from the moist substrates, saved them from being desiccated. Results obtained in the laboratory indicate that green toads can withstand far more stressful conditions than those prevailing in their natural habitat.

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ANNOUNCEMENT

EIGHTH WORLD CONGRESS ON ANIMAL, PLANT AND MICROBIAL TOXINS

University of Newcastle-upon-Tyne, England, 11–16 August 1985

Sponsored by The International Society on Toxinology

Arrangements for this meeting are now in hand. A suite of lecture theatres in a modern teaching block has been reserved and accommodation for 500 participants is available a few minutes walk from the teaching block.

The programme will concentrate on growth areas in toxin research, and on the clinical problems resulting from poisoning.

Dates to remember: July 1984, second notice to members; January 1985, final notice to members; and March 1985, registration and abstracts (to be sent to The Secretariat).

Intending participants are invited to contact: The Secretariat, Eighth World Congress on Animal, Plant and Microbial Toxins, Muscular Dystrophy Group Research Laboratories, Newcastle General Hospital, Westgate Road, Newcastle-upon-Tyne NE4 6BE, England.

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REVIEW

CARDIOMYOPATHY IN TURKEYS

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Abstract—1. Round heart disease (RHD) is a spontaneous occurring cardiomyopathy in fowl affecting primarily inbred, small broad-breasted white strains of turkeys.

2. Etiology of RHD is unknown but factors implicated include genetic, management, enzyme deficiencies, viral, immunologic, and metabolic.

3. Investigations of proposed etiologic factors are reviewed.

4. Furazolidone (FZ)-induced cardiomyopathy is indistinguishable from spontaneous RHD, but it remains to be shown that both are mediated by the same mechanism(s).

5. Studies to date suggest that FZ-induced cardiomyopathy is an exaggeration of the spontaneous condition.

INTRODUCTION

Round heart disease (RHD) is a spontaneously occurring cardiomyopathy in fowl. Clinically, the disease is characterized by sudden death of what seem to be normal birds. Various names suggesting either the appearance of the affected heart or the nature of the symptoms have been used to describe the condition.

The disease was first reported in chickens on 38 New Zealand farms (Fischel, 1946). Fischel termed the condition enzootic fatal syncope or toxic heart degeneration. In 1947, the disorder in chickens, resulting in stunted growth and death, was described by several investigators (Blaxland and Markson, 1947; Luke, 1947). Luke was first to use the term RHD because of the post-mortem appearance of the heart. A spectacular rise in the incidence of the disease was reported in Scotland and England in the early 1950s (Wilson and Siller, 1954).

Early investigations of the syndrome in chickens revealed the selectivity of the disease for certain breeds, a seasonal incidence, and age with pullets most commonly affected (Wilson and Siller, 1954). In some flocks, the birds were affected indiscriminately. Later studies showed that there was an association between RHD and the built-up litter system of poultry management (Wilson, 1957). However, studies conducted to determine the causal agent were unsuccessful.

SPONTANEOUS CARDIOMYOPATHY IN TURKEYS

In 1962, RHD was identified in turkeys (Magwood and Bray, 1962). The syndrome is characterized by cardiomegaly due to extensive dilatation of the ventricles, congestive heart failure, and increased mortality in young poults (Sautter *et al.*, 1968; Noren *et al.*, 1971b; Gough *et al.*, 1981). Typically the affected poult has a labored, gasping breathing when handled and exhibits signs of dyspnea with levels of exercise at which normal poults show no signs of distress. At necropsy, the affected poult characteristically has a greatly enlarged heart approximating a sphere in shape compared to the cone-shaped heart of the normal poult (Fig. 1). The distension of the heart is so striking in advanced cases that it may occupy an abnormally large portion of the thoracic cavity. Ascites, pulmonary edema, and hydropericardium are commonly associated with the syndrome in turkey poults.

The etiology of the disease syndrome in turkeys is unknown. Published reports indicate that RHD is more common in males than females (Magwood and Bray, 1962; Hunsaker *et al.*, 1971). Generally speaking, the condition appears to be associated with some form of stress, such as chilling, excessive inhalation of various fumes, dietary deficiencies, and problems of early brooding (Sautter *et al.*, 1968). Investigations of RHD have suggested a number of etiologies: genetic, management factors, α-1-antitrypsin deficiency, viral, immunologic, and metabolic. There is little doubt that a genetic basis is involved in RHD. In susceptible poults, it appears that some form of stress interacts with the genetic defect(s).

The round heart syndrome occurs most frequently in inbred, small broad-breasted white strains of turkeys (Hunsaker, 1971; Einzig *et al.*, 1972). An experimental breeding flock has been established with progeny having a grossly identified incidence of RHD of up to 26% (Jankus *et al.*, 1973). Breeding trials suggest the disease has a familial mode of inheritance. Mortality in the experimental flock was reported to be 14% during the first 4 weeks post-hatching (Hunsaker, 1971). Maximum mortality of spontaneous RHD in turkeys was 22% with the majority of the deaths occurring at 2 weeks of age (Noren *et al.*, 1971b; Jankus *et al.*, 1973). This mortality rate is considerably greater than the rate of 6% in commercial flocks relatively free of the disease. By 1 month of age, about half of the inbred poults demonstrate an evident cardiomegaly. Clinically, the affected

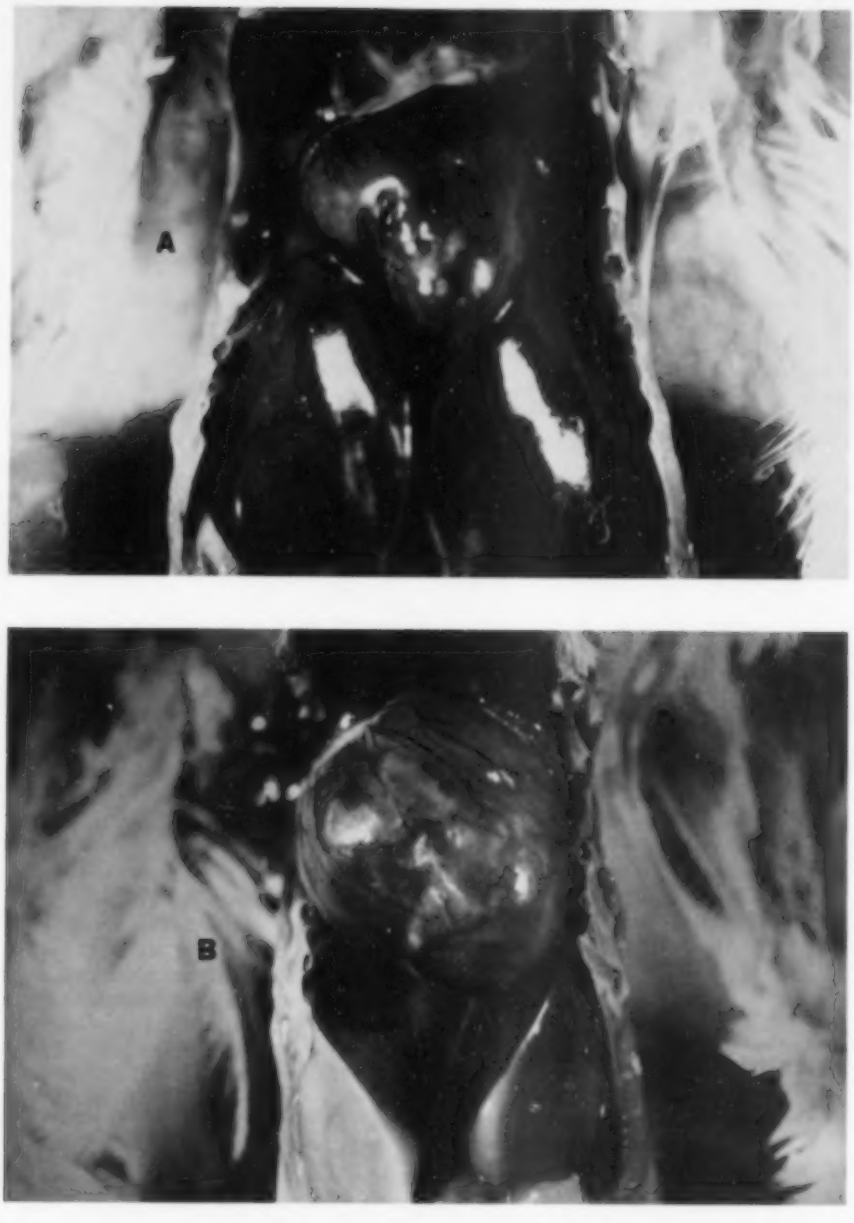

Fig. 1. Hearts from 5-week-old turkey poults. A: control heart with no dilatation. B: heart from a furazolidone (FZ)-fed poult demonstrating ventricular dilatation (×1.5).

poults have an unhealthy appearance with ruffled feathers and drooping wings. The disease has been described as having two phases (Jankus *et al.*, 1973). The acute phase occurs during the first 2 weeks of life and is characterized by pancarditis, focal interstitial myocarditis, and high mortality. A wide spectrum of cardiac dilatation, ventricular hypertrophy, and left ventricular endocardial fibroelastosis characterizes the chronic phase of the disease. During this phase, death is usually due to congestive heart failure.

The morphology of the myocardial cells in affected poults has been described by a number of investigators (Sautter *et al.*, 1968; Noren *et al.*, 1971b; Gough *et al.*, 1981). Sautter *et al.* (1968) reported that no consistent lesions were present in either the heart or the liver of afflicted turkeys at various ages. In poults up to 8 weeks of age the most consistent findings were marked myocardial congestion, hemorrhage, and epicardial fibrosis. On the other hand, the myocardium of mature (20-week-old) turkeys did not exhibit the intense congestion observed in poults. In the older birds, foci of acellular collagenous repair

were noted. In contrast to the younger poults, adult birds had minimal changes in the epicardium but a pronounced fibroelastosis in the endocardium.

Light microscopic findings of hearts from young poults from a flock of small white broad-breasted turkeys with a known high incidence of RHD were reported by Noren *et al.* (1971b). Their examination of abnormal hearts revealed focal interstitial and perivascular infiltrates of lymphocytes and occasional eosinophils in the myocardium, epicardium, and endocardium. These infiltrates were observed between 1 and 4 weeks post-hatch. Turkeys older than 6 weeks showed no evidence of the myocarditis seen in the younger poults. However, in poults older than 3 weeks, a gradual increase in the deposition of fibroelastic tissue was noted in the endocardium of the left ventricle.

In a recent study, Gough *et al.* (1981) were unable to detect specific pathologic lesions in affected poults. Histologic alterations such as pale and wavy fibers with indistinct cross-striations, fragmentation of sarcoplasm, disorientation of nuclei, and presence of intracellular vacuoles were discounted as artifacts of tissue preparation.

Vectorcardiography (Jankus *et al.*, 1971) and electrocardiography (Hunsaker *et al.*, 1971; Jankus *et al.*, 1971) have been used to describe physiologic changes in RHD. In normal poults at 2 days of age, the orientation of the vectorcardiogram (VCG) was reported to be left, caudad, and dorsad. By 54 days of age, the VCG orientation was described as being "fixed" near the mid-sagittal plane. Lead I was said to be nearly isoelectric, the S wave predominated on leads II and III, and a positive T wave was present (Hunsaker *et al.*, 1971). In turkeys with RHD, the orientation of the VCG was similar to that of a 2-day-old poult, but was greater in magnitude (Jankus *et al.*, 1971). Hunsaker *et al.* (1971) showed that the R–S axis shifted from -85° in the normal poult to $+70^{\circ}$ in the poult with RHD. In the latter, the S wave was small or absent and the R wave was greater than that from control poults on all leads. These findings were confirmed by Jankus *et al.* (1971). The latter investigators suggested that the increase in the amplitude of the R wave be used as the primary criterion for identifying turkeys with RHD. Utilizing this technic, poults could be categorized into one of four groups: normal (N), normal transitional (NT), round heart transitional (RT), and round heart (R). The basic ECG technic was modified by Czarnecki and Good (1980) to permit the identification of poults in the early stages of a developing cardiomyopathy. These poults were designated as normal with high voltage on lead I (N_H). To date, the basic ECG technic with the recent modification (Fig. 2) is the best diagnostic tool for identifying afflicted poults.

A number of hemodynamic factors have been investigated during the chronic phase of the disease (Einzig *et al.*, 1972, 1980). Poults in this stage were reported to have reduced cardiac output, elevated right and left ventricular filling pressures, and systemic hypotension. Atrial and ventricular blood flow values were similar in both control and RH poults, but the left ventricular subendocardial/subepicardial blood flow ratios were decreased in RH poults when compared to those in control birds. In the RH poults, echocardiography revealed evidence of left ventricular dysfunction characterized by left atrial and left ventricular dilatation and a markedly reduced left ventricular shortening fraction. These data suggested that relative underperfusion of left ventricular subendocardial muscle could be responsible for the development of endocardial fibroelastosis observed in older birds during the chronic phase of the disease.

Published data on the heart rate of afflicted turkeys are conflicting. In one study, the heart rates of RH poults, when compared to control poults, were found to be lower at 19 days of age and higher at 50 and 57 days of age (Jankus *et al.*, 1971). However, in another study, heart rates of affected birds were reported to be similar to those of unaffected turkeys (Einzig *et al.* 1972). These investigators, also, observed decreases in aortic pressure and increases in the mean right atrial and mean pulmonary arterial wedge pressures. No significant differences in either the mean systemic or mean pulmonary vascular resistance were seen, but the ratio of the two was significantly lower due to a slight increase in mean pulmonary vascular resistance. In turkeys that had recovered from RHD, these parameters were similar to those of controls except that a decrease in aortic pressure was still noted. Data obtained from dye dilution curves from a recovered RH poult indicated a distorted delay slope consistent with valvular regurgitation, left to right ventricular shunting, or cardiac failure. Einzig *et al.* (1972) postulated that the distorted delay slope of the indicator dilution curve was

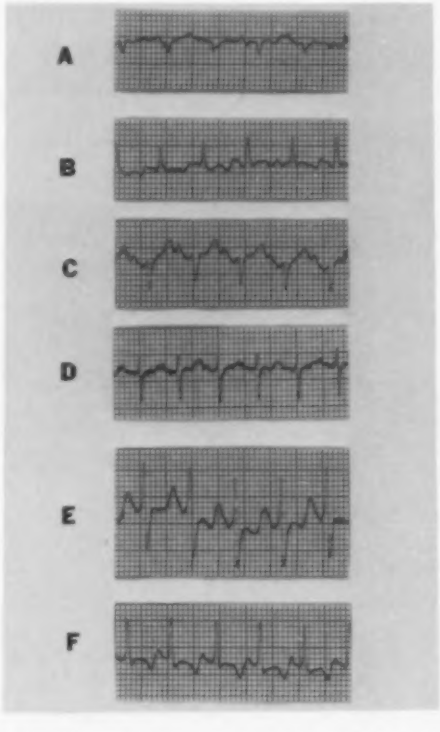

Fig. 2. Basic ECG patterns on lead I (A and B) and lead II or aVF (C–F). A: normal (N). B: voltage > 0.1 mV in R wave (N_H). C: normal (N). D: normal transitional (NT). E: round heart transitional (RT). F: round heart (R).

most likely due to mitral insufficiency since a septal defect was not discernible anatomically.

Calcium bindings, calcium uptake, and calcium stimulated ATPase have been studied in the myocardium of RH turkeys using preparations of isolated sarcoplasmic reticulum (Staley *et al.*, 1981). Data from this study showed that calcium binding was increased during the first month post-hatching in control poults but not in those with RHD. Calcium uptake increased in both groups, but this increase occurred at a significantly slower rate in RH birds. Calcium stimulated ATPase was similar in both groups throughout the first month, but decreased thereafter in birds with RHD.

Chemical substances, altered following myocardial and/or hepatic injury, have been demonstrated in some poults with spontaneous RHD. Several investigators reported an α-globulin deficiency in Nicholas turkeys with RHD (Neumann *et al.*, 1973; Meirom *et al.*, 1974, 1975; Rattner, 1976). This deficiency was associated with lowered serum trypsin inhibitory capacity (TIC), hypoproteinemia, and the presence of PAS-positive globules in hepatocytes. The TIC deficiency was postulated by Rattner (1976) to be hereditary in turkeys and was thought to predispose the poults to RHD which may result from an interaction between genetic and environmental factors. A study by Staley *et al.* (1978) failed to support these reports since diastase-resistant PAS-positive globules could not be demonstrated in the livers of turkeys from the inbred flock irrespective of whether the birds had cardiac enlargement. Since the etiology of RHD in the inbred flock is not related to an absence or decrease of protease inhibitors, it is highly unlikely that a decrease in serum proteins results in this cardiomyopathy.

Conflicting data have been presented on the influence of high levels of dietary salt or sodium on the development of RHD in turkeys (Dewar and Siller, 1971; Morrison *et al.*, 1975; Leeson *et al.*, 1976). Occasionally, reductions in dietary salt levels are recommended in field outbreaks of the disease. In those cases, Leeson *et al.* (1976) suggest that minimum supplemental salt levels of 0.2–0.3% should be fed turkeys on corn-soybean diets.

A viral etiology has been suspected by virtue of the demonstration of C-type viral particles in the myocardium by electron microscopy (Staley *et al.*, 1972). The C-type viruses include the leucosis-sarcoma and reticuloendotheliosis avian viruses, but the serum of affected poults lack titers to these agents. Furthermore, inoculation of 1-day-old poults with material from infected tissues has failed to induce the disease. Although the hypothesis of a viral etiology remains attractive for RHD since viruses have been implicated in human myocarditis, corroboration of the C-type virus particles remains to be achieved.

Improved management technics are believed to be responsible for the apparent decrease in reported outbreaks of the spontaneous cardiomyopathy in turkeys. However, in an informal survey of a number of commercial poults over a period of 18 months, we noted that 20–25% of these poults exhibit an abnormal ECG pattern at 2 weeks of age consistent with that reported for poults with RHD. Findings such as these strengthen the supposition of a genetic basis for the disease.

FURAZOLIDONE-INDUCED CARDIOMYOPATHY IN TURKEYS

The decrease in number of spontaneously affected poults necessitated the development of a method for producing a similar syndrome experimentally. This was first accomplished by Jankus *et al.* (1972) who noted that furazolidone (FZ), a nitrofuran, was toxic in turkey poults at concentrations as low as 300 ppm when fed continuously for 5 weeks post-hatching and

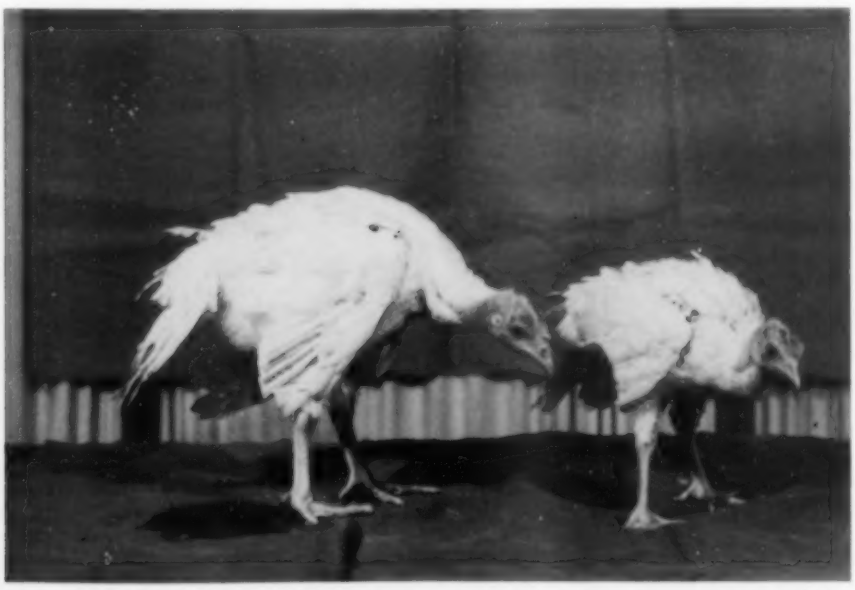

Fig. 3. 5-week-old poults from a single hatch. Control poult is on left. Poult on right has FZ-induced cardiomyopathy.

was associated with death, cardiac dilatation, and ascites. At a dose of 500 ppm, a mortality rate of 80% was reported while at doses of 700 ppm or higher, all of the poults died. Czarnecki *et al.* (1974a) confirmed the toxicity of FZ at doses as little as 300 ppm and determined the effect of graded doses of FZ on body weight and development of cardiomyopathies in young turkey poults. At toxic levels, the growth rate of unaffected FZ-fed poults paralleled that of control poults. These reports of FZ toxicity in young turkey poults were substantiated by Jensen *et al.* (1975). The drug-induced condition is indistinguishable from spontaneous RHD, but it has yet to be demonstrated that both conditions are mediated by the same mechanism(s). Numerous similarities suggest a common pathogenic pathway.

The use of FZ to induce the RH syndrome has been a boon to investigators. Studies in our laboratory (Czarnecki and Good, 1980) have shown that 25–40% of the poults develop an N_H ECG pattern within 5 days when fed FZ at a dose of 700 ppm beginning at 2 weeks of age. The majority of these poults develop the typical NT to R ECG pattern, characteristic of the RH syndrome, by the end of the second week following the institution of the drug regimen. In affected poults, body growth is significantly depressed being about half that of control (normal) poults (Fig. 3). Cardiomyopathy observed most commonly is a combined right and left ventricular dilatation (Fig. 4). Both of these observations are consistent with those reported for spontaneous RHD (Hunsaker *et al.*, 1971).

A recent study has clarified the sequence of development of cardiac dilatation (Czarnecki *et al.*, 1983a). The investigators noted that FZ-induced cardiomegaly is due first to a dilatation of the right ventricular lumen, followed by a dilatation of the left ventricular lumen, and finally by continued dilatation of both venticular lumina. Cardiomegaly observed did not appear to involve hypertrophy since the area of heart muscle in both ventricular walls was significantly less than that seen in control poults. The significant decrease in the ratio of heart muscle to total heart in affected poults, compared to control poults, was further evidence that hypertrophy plays little or no role in the development of FZ-induced cardiomyopathy. Data from this study indicate that changes in morphology may be responsible for the altered ECGs. It was hypothesized that an increase in intracavitary blood occurring secondarily to cardiac enlargement may be the cause for the decrease in QRS voltage and congestive heart failure noted in advanced cases.

In addition to its demonstrated toxicity to cardiac muscle, FZ produces anorexia in turkey poults (Ali and Bartlet, 1982). The reduced feed intake results in a depression of growth as reported in an earlier study (Czarnecki *et al.*, 1974a). However, there is no reduction in heart weight since FZ significantly increases the ratio of heart weight to body weight and increases myocardial moisture content (Czarnecki and Jankus, 1975). This cardiac enlargement is irreversible, and it cannot be accounted for by an increase in moisture content since removal of FZ from the diet results in the return of moisture content to normal levels. The reduced feed intake of poults on the FZ diet is not apparent during the first 2–3 days on the regimen indicating this was not due to a distaste for the drug but rather to an effect which developed during this period. Following withdrawal of the drug, feed intake and rate of growth are increased (Czarnecki *et al.*, 1974a; Ali and Bartlet, 1982). This demonstrates that the effects of FZ on body growth are reversible lending credence to the supposition that the effects of FZ are varied, but that the most damaging effect occurs in the myocardium.

A number of observations suggests that the mechanism of FZ-induced cardiomyopathy may be metabolic in nature. For instance, it has been demonstrated that levels of glycogen in the myocardium of

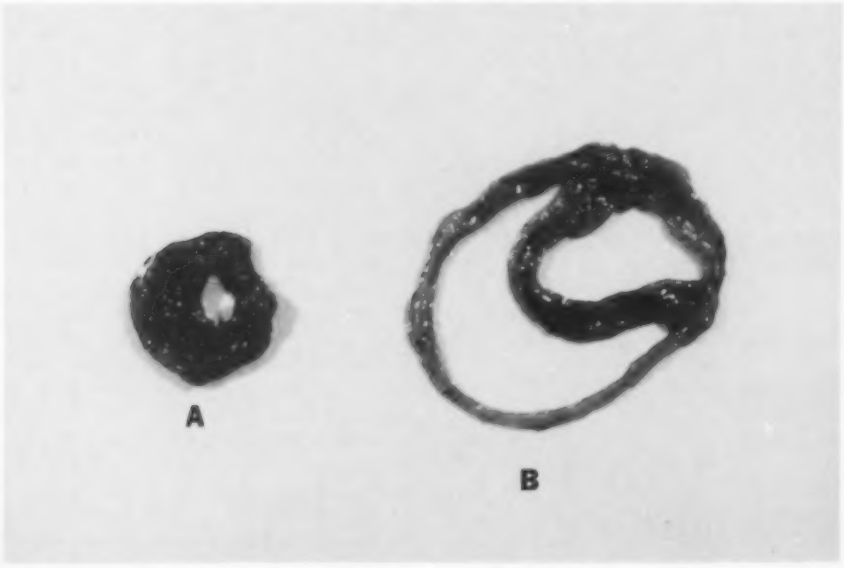

Fig. 4. Cross-sections of heart from 5-week-old poults. A: control heart with no dilatation. B: heart from poult with FZ-induced cardiomyopathy showing biventricular dilatation (×2).

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afflicted turkey poults are significantly increased (Czarnecki *et al.*, 1974b; Czarnecki *et al.*, 1975; Czarnecki and Evanson, 1980). The increase was noted as early as one week after the poults were placed on the drug regimen. Removal of the drug from the diet results in the restoration of normal myocardial glycogen levels. In spontaneous RHD, myocardial glycogen levels, also, are significantly elevated above control values (Czarnecki and Jankus, 1974). Unaffected poults from the experimental flock with a high incidence of RHD have high concentrations of myocardial glycogen suggesting that altered glycogen metabolism may be related to the development of this syndrome.

The supposition that RHD is associated with a type of glycogen storage disease is enhanced by the similarities between RHD in turkey poults and Pompe's disease in human infants. In the latter condition, a genetic abnormality has been identified that leads to a deficiency of α,1,4-glucosidase. A study in our laboratory demonstrated that normal levels of this lysosomal enzyme were present in both affected and unaffected poults (Czarnecki *et al.*, 1978b). These data were corroborated by a subsequent investigation (Czarnecki *et al.*, 1978a) in which it was shown that abnormal accumulations of myocardial glycogen in affected poults were comprised of glycogen with normal characteristics (i.e., per cent of branching and number of glucose residues/segment). Based on these observations, it was hypothesized that RHD more closely resembled an adult form of Type II glycogenosis. The metabolic defect in the latter condition has not been identified, but it has been postulated that the basic abnormality is due to an absence or deficiency of a second enzyme (Garancis, 1968).

Accumulation of cytoplasmic glycogen in the myocardial cell appears to be the result of an imbalance between synthesis and degradation. Inhibition of glycogen degradation seems to be the most plausible explanation since glycogen synthesis is normal (Czarnecki *et al.*, 1978a). Faulty utilization of glycogen and/or inhibition of activity of glycogenolytic enzymes is thought to result in excessive accumulation of myocardial glycogen. It is unclear whether this contributes to the development of the cardiomyopathy or is the sequel to the disturbances in myocardial metabolism. A lack of significant differences in blood serum glucose levels in affected and unaffected poults indicates that FZ is not exerting an influence on hormones involved in the regulatory mechanisms of circulatory glucose concentration.

Serum proteins have been shown to be depressed in FZ-induced cardiomyopathy (Staley *et al.*, 1978; Czarnecki *et al.*, 1981). In the study by Staley *et al.* (1978), evidence was presented that in the turkey poults, FZ produces primary hepatic damage which is reflected in the depression of total serum proteins, including TIC capacity, and that these alterations are superimposed on the heart in susceptible poults. Thus, the decrease in serum TIC may be secondary to a nonspecific reduction of all serum proteins, probably due to liver damage caused by FZ or its metabolites. As with the accumulation of myocardial glycogen, it would seem that deficiencies and/or lowering of serum proteins are the result of faulty metabolism.

Taurine levels have been examined in the brain, skeletal muscle, and myocardium of FZ-fed poults (Schaffer *et al.*, 1981, 1982). These investigations demonstrated that taurine levels are depressed significantly in the heart, but are not altered in the brain or skeletal muscle suggesting a selective toxicity of FZ to the myocardium. The return of myocardial taurine to normal levels following the removal of FZ from the diet is further evidence of the drug's influence on cardiac metabolism.

Myocardial morphologic alterations in FZ-induced cardiomyopathy and spontaneous RHD are similar to those commonly associated with cardiac hypertrophy. These include increased accumulation of sarcoplasmic glycogen, changes in various cell organelles most notably the mitochondria and sarcoplasmic reticulum, appearance of nuclear and intramitochondrial glycogen, alteration of Z-band material with a concomitant increase in number of sarcomeres, and an increase in extracellular space with a loss of intercellular contact (Czarnecki, 1980). Volumetric densities of mitochondria and myofibrils are depressed significantly during the development of FZ-induced cardiomyopathy (Czarnecki and Grahn, 1980), but this has not been investigated in spontaneous cases since sufficient numbers of the latter have been unavailable for a statistical study.

Alterations in myocardial morphology and plasma concentrations of enzymes (Avidan *et al.*, 1977; Czarnecki, 1980; Czarnecki *et al.*, 1981) known to be altered following myocardial and/or hepatic injury suggest damage to the sarcolemma. Evidence for this was provided in a recent study that showed sarcolemma isolated from hearts of afflicted poults exhibited elevated calcium binding capacity (Schaffer *et al.*, 1981), a property commonly associated with calcium overload. However, data from a subsequent study (Czarnecki and Evanson, 1983) indicated that calcium overload plays no significant role in FZ-induced cardiomyopathy.

Immunologic mechanisms that might be involved in the development of spontaneous RHD and FZ-induced cardiomyopathy have received some attention. Treatment with cortisone acetate was reported to be effective in significantly reducing the mortality during the first few weeks post-hatch in poults from an inbred flock of turkeys with a known high incidence of spontaneous RHD (Noren *et al.*, 1971a). In a subsequent study, Staley *et al.* (1975) observed that 2.0 mg of cortisone acetate administered daily to poults from the inbred flock significantly reduced mortality during the first 5 weeks post-hatch, delayed the time at which peak mortality occurred by about 6 days, and decreased the incidence of cardiac dilatation. Additionally, this investigation noted that mortality was significantly reduced during the first 10 days of age in bursectomized and cyclophosphamide-treated poults, and the incidence of cardiac dilatation was significantly reduced in bursectomized poults when compared to control poults. Another study indicated that administration of 2.5 mg of cortisone acetate intramuscularly daily significantly reduces the incidence of FZ-induced cardiomyopathy and delays the onset of altered ECG patterns by approximately

1 week, but has little or no effect on mortality or the development of spontaneous cardiomyopathy during the first 2 weeks post-hatch (Czarnecki *et al.*, 1980). More recently it was reported that cortisone acetate, fed at a dose of 500 mg/kg of ration beginning at 1 week of age, decreased mortality but had little effect on the development of FZ-induced cardiomyopathy (Czarnecki *et al.*, 1983b). Further work is needed in this area, but the possibility that the immunologic response is a common pathway involved in both spontaneous RHD and drug-induced cardiomyopathy remains an interesting speculation.

SUMMARY

The etiology of spontaneous RHD and the mechanism of FZ-induced cardiomyopathy remain an enigma. Several cardiac biochemical effects of FZ have been proposed, including (a) inhibition of monoamine oxidase activity (Powers *et al.*, 1983), (b) altered carbohydrate metatabolism (Czarnecki and Jankus, 1974; Czarnecki *et al.*, 1974b, 1975, 1978a; Czarnecki and Evanson, 1980), (c) altered protein metabolism (Staley *et al.*, 1978; Simpson *et al.*, 1979), and (d) decreased levels of myocardial taurine (Schaffer *et al.*, 1981, 1982). However, the evidence to date indicates that these effects are secondary to the basic deficiency in susceptible poults and/or the toxic insult induced by FZ.

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DYNAMICS OF OOCYTE DEVELOPMENT AND RELATED CHANGES IN SERUM ESTRADIOL-17β, YOLK PRECURSOR, AND LIPID LEVELS IN THE TELEOSTEAN FISH, *LEPTOCOTTUS ARMATUS*

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Abstract—1. The dynamics of ovarian development in *Leptocottus armatus* were studied. Seasonal changes of serum estradiol-17β, serum vitellogenin, serum total lipids and visceral lipid reserves were monitored.

2. Two to four distinct size classes (clutches) of oocytes occurred in the ovary preceding and during the spawning period. One of these clutches contained oocytes in the vitellogenic or post-vitellogenic phase, a second clutch contained oocytes "arrested" in the cortical alveoli stage and a third clutch contained non-yolky oocytes. Only one clutch was oviposited in the annual spawning season.

3. Estradiol levels increased coincident with the appearance of yolky oocytes in the ovary. Serum estrogen levels were highest during the period of ovulation and spawning. During the spawning period estradiol titers were not significantly correlated with oocyte size or gonosomatic index.

4. Serum vitellogenin levels increased in parallel with oocyte size and estradiol titers. Vitellogenin levels were relatively low during the early portion of the spawning period, becoming elevated again towards the end of the spawning period. In concordance with ovarian regression, serum vitellogenin levels declined and remained low during the ovarian quiescence phase. Neither GSI nor serum estradiol titers correlated significantly with vitellogenin levels.

5. With initiation of ovarian recrudescence visceral lipid reserves began to decline and continued to decline through the vitellogenic and spawning phases. Visceral lipid reserves were inversely correlated with ovarian GSI. Serum lipid levels were high during ovarian recrudescence, remaining elevated through the spawning period. During the phase of ovarian quiescence serum lipid levels were low and visceral lipid reserves were high. Visceral lipid reserves showed an inverse correlation to serum estradiol titers.

INTRODUCTION

That reproduction is a seasonal event among a majority of teleostean fishes occurring in temperate latitudes is well documented (e.g., deVlaming, 1974). Gonadal weight variations are known to reflect the annual spawning cycles, yet detailed examination of oocyte development dynamics are not common. Estrogens undoubtedly play critical roles in teleostean reproduction; nonetheless, seasonal profiles of serum estradiol-17β levels have been recorded mostly for salmonid species. Of particular value would be investigations which attempt to correlate annual variations of serum estradiol levels with a thorough analysis of oocyte development dynamics. The secondary growth phase of oocytes among teleosts is gonadotropin dependent (deVlaming, 1983). Yolk accumulation is the hallmark of the secondary growth phase, and the phosphoprotein and lipoprotein yolk material is of extraovarian origin. Moreover, estrogens induce the hepatic synthesis of the yolk precursor, vitellogenin (e.g., deVlaming *et al.*, 1980). Reports of seasonal variations of serum vitellogenin titers among teleostean fishes are infrequent. Although estrogen promotes vitellogenin synthesis, few studies have demonstrated correlations between seasonal changes in serum levels of estradiol and vitellogenin.

Attempts have been made to associate seasonal variations of body lipid reserves with gonadal cycling among piscine species. Indeed, vitellogenin contains considerable lipid (e.g., deVlaming *et al.*, 1980), and eggs, especially of marine teleosts, possess lipid inclusions. Estrogen has been implicated in the mobilization of lipid (e.g., deVlaming *et al.* 1977a,b). The relationship of lipid mobilization to oocyte growth, as well as the endocrine control of this process, requires more extensive study among teleostean fishes.

Although cottid fishes are widely distributed in both marine and freshwater environments of the world, little is known concerning the dynamics of ovarian development or the reproductive endocrinology of this taxonomic group. The staghorn sculpin, *Leptocottus armatus*, ranges in the coastal waters of the eastern Pacific Ocean and connecting seas and rivers from Kodiak Island, Alaska (57°15′N) to San Quentin bay, Baja, California (30°15′N) (Jones, 1962). *Leptocottus* is a demersal fish of shallow waters

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and frequents bays, inlets and sounds. This is a truly euryhaline species in that individuals have been collected in salinities from fresh water to seawater (Jones, 1962).

Our aim was to analyze the seasonal dynamics of ovarian development, as well as some of the associated endocrine and metabolic events in the staghorn sculpin. In addition to tracking the annual pattern of oocyte development, we also assayed serum estradiol-17β and phosphoprotein (vitellogenin) levels, serum lipid levels, and visceral lipid stores. Attempts were made to relate these paramaters.

MATERIALS AND METHODS

Staghorn sculpins, *Leptocottus armatus*, were collected on a monthly basis by trawling in San Pablo Bay, California. San Pablo Bay connects broadly to San Francisco Bay. During the period when water temperatures were highest (i.e., July and August), few of these fish could be collected. Fish were killed and samples taken within 36 hr of collection. Body weights were taken to the nearest 0.01 g and blood samples drawn by cardiac puncture into heparinized syringes. The paired ovaries were removed and weighted to the nearest 0.001 g. Contents of the coelomic cavity (stomach, liver, intestinal tract and mesenteries) were removed; following removal of the gut contents (which were weighed to the nearest 0.01 g) the coelomic organs were frozen for lipid analysis at a later time. The gonosomatic index was calculated as a percentage of body weight; specifically, weight of the ovaries was divided by the corrected body weight (i.e., body weight less the weight of the gut contents and ovaries). Once the ovaries were weighed ovarian pieces or isolated follicles were fixed in 4% formalin in phosphate buffered saline. Within the ovarian follocle the oocyte is surrounded by an acellular vitelline envelope, a follicular epithelium, a thecal layer, and the surface epithelium. Follicular diameters were measured in the fixed tissue using a dissecting microscope equipped with an ocular micrometer. Although measurements are of follicles we will, hereafter, refer to oocyte diameters. In *Leptocottus* there can be 1–4 clutches (size groups) of oocytes in an ovary. Remarkable uniformity exists in the diameter of oocytes in each clutch, especially the leading clutch (group of oocytes with the greatest diameter). The diameter of oocytes in all clutches for each ovary was determined; means for each clutch could then be determined for all females in the monthly sample. Some of the oocytes were also processed for histological examination and for relating oocyte diameter with stage of development. The formalin-fixed ovarian tissue to be used for histology was washed in 0.1 M phosphate buffer (pH 7.4), dehydrated, and embedded in JB4 plastic (Polysciences Co.). Sections, 2–3 μm in thickness, were placed on glass slides and stained with hemotoxylin and eosin.

Visceral tissue (contents of coelomic cavity) was lyophilized prior to lipid extraction. In *Leptocottus*, as in most other teleosts, lipid is deposited in the mesenteries and liver. Hereafter the lipid stores in these tissues will be referred to as the visceral lipid stores. The lyophilized visceral tissue was transferred to a desiccator jar, samples being weighed rapidly. Following this weighing, samples were extracted with ethyl ether for 120 hr and with acetone for 48 hr at the boiling point of the solvents in a goldfish extraction apparatus (Laboratory Construction Company). The extracted samples were dried under vacuum and then weighed. Lipid content of the tissues was then calculated by the pre- and post-extraction weight difference. For verification, lipid in the extraction solvents was gravimetrically determined. Protein in the visceral tissues was assayed according to the Kjeldal technique. Visceral lipid reserves are expressed as mg lipid per g protein.

Blood collected into heparinized syringes was placed into microfuge tubes containing 30 μl of the proteolytic enzyme inhibitor, Trasylol (Aprotinin—Mobay Chemical Corporation). After thorough mixing the tubes were placed on ice for 30–60 min. The protease inhibitor was used for protection against vitellogenin degradation (see deVlaming *et al.*, 1980). Plasma was collected following centrifugation of the blood. Clots formed in plasma samples before or during storage at -20°C, or in the thawing prior to assays. Hereafter we will refer, therefore, to concentration of factors in serum. Serum vitellogenin titers were estimated as alkali labile phosphorus (P). In all teleosts examined to date vitellogenin represents the sole serum phosphoprotein. Alkali labile P levels were determined by adding 1 ml of 12% TCA to 200 μl of serum. After centrifugation the supernatant was poured off and the pellet washed twice with one ml of an ethanol–ether mixture (3:1) followed by a wash with ether alone. The remaining pellet was then resuspended in 0.5 ml of 1 M NaOH and placed in a 100°C H_2O bath for 15 min to liberate the protein bound phosphorus. Once cool, the mixture was neutralized with glacial acetic acid, and protein was again precipitated with 0.5 ml of 40% TCA solution. P was then determined by the molybdate reaction described by Lowry and Lopez (1946). Serum protein was determined by the technique of Lowry *et al.* (1951) using 10 μl of serum and BSA as a standard. The spectrophotometric procedure of Frings and Dunn (1970) was employed for the estimation of serum total lipid levels; 100 μl of serum was used in this technique which is based on the sulphophospho-vanallin reaction.

Serum estradiol-17β levels were quantified by radioimmunoassay (RIA) according to the procedure routinely used by Barkley *et al.* (1979). The specificity, validity and reliability of the assay have been reported (Boilert *et al.*, 1973). The anti-estradiol serum was a gift from Dr L.-E. Edqvist and was produced in sheep against E_2 conjugated at C-6 to bovine serum albumin. The E_2 antiserum was diluted 1:500,000 in a 0.1% Na azide, 0.9% NaCl and 0.1% gelatin 0.1 M phosphate buffer (PSA). This dilution of the antiserum allowed 50% binding of the labelled E_2 in the absence of radioinert steroid. Solvent, water and PSA blanks were consistently less than or equal to 3 pg/ml. Serum samples were extracted with 5 volumes of diethyl ether (freshly opened), the extracts were immersed in an acetone/solid CO_2 bath, the ether phase placed in a culture tube, and the extracts were evaporated to dryness. This method extracted 95–98% of the serum E_2. The dried extracts were resuspended in PSA. Separation of the free from the bound steroid was performed using a 1 ml mixture of Dextran T70 (0.25 g/l) and charcoal (2.5 g/l).

RESULTS

Oocyte development in *Leptocottus armatus* is consistent with the generalized pattern among oviparous teleosts (deVlaming, 1983). Moreover, oogonial proliferation is followed by the primary growth phase in which germinal vesicle (nucleus) size increases and multiple nucleoli appear (perinucleolar stage). During this phase the nuclear to cytoplasm ratio decreases as the oocyte volume increases. The early secondary growth phase of oocytes is characterized by accumulation in the ooplasm of cortical alveoli (sometimes termed "yolk vesicles"—Fig. 1A). In later stages of the secondary growth phase cortical alveoli lie subjacent to the oocyte membrane (Figs 1B and D). The accumulation of yolk is responsible for the enormous enlargement of oocytes during the secondary growth phase. Oocytes of *Leptocottus* accumulate yolk proteins in yolk spheres having a fluid consis-

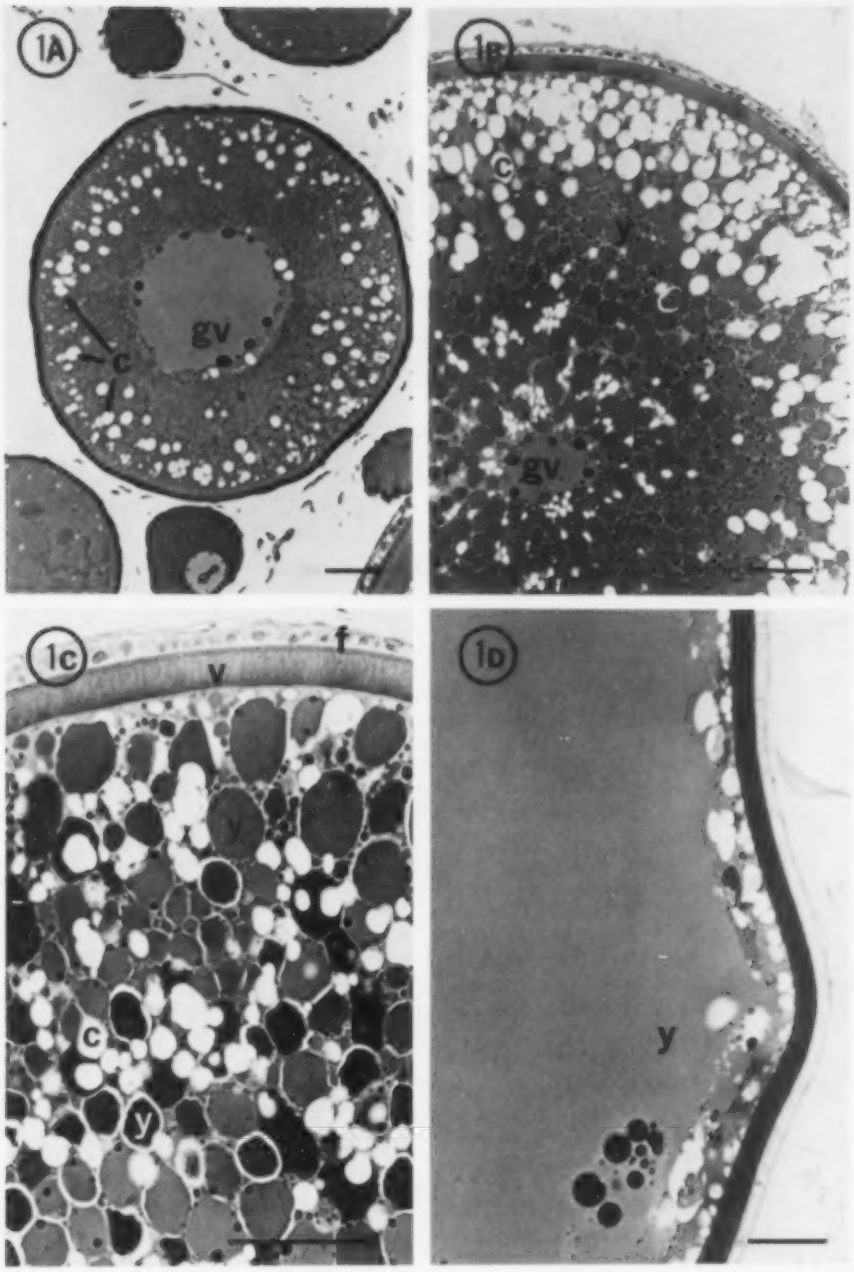

Fig. 1. Ovarian follicles of *Leptocottus armatus* in different stages of development. Bar = 50 μm. (A) A follicle containing a "yolk vesicle" stage oocyte, with numerous cortical alveoli (c). Typically, the germinal vesicle (gv) contains many peripherally-located nucleoli. (B) A vitellogenic follicle containing numerous yolk spheres (y) and cortical alveoli (c). (C) A higher magnification of a vitellogenic follicle. Follicular epithelium (f); vitelline envelope (v); yolk spheres (y); cortical alveoli (c). (D) A late vitellogenic or early maturational stage follicle containing a continuous mass of fluid yolk (y).

tency (Figs 1B and C). Towards the end of vitellogenesis and prior to ovulation the oocyte germinal vesicle migrates to a peripheral position and disappears. Concomitant with this process the yolk spheres fuse to yield a continuous mass of fluid yolk (Fig. 1D); small lipid-filled vesicles also coalesce to form larger lipid droplets.

Our data suggest that female staghorn sculpins do not become sexually mature until standard length is 125 mm. The ovaries of immature females contain

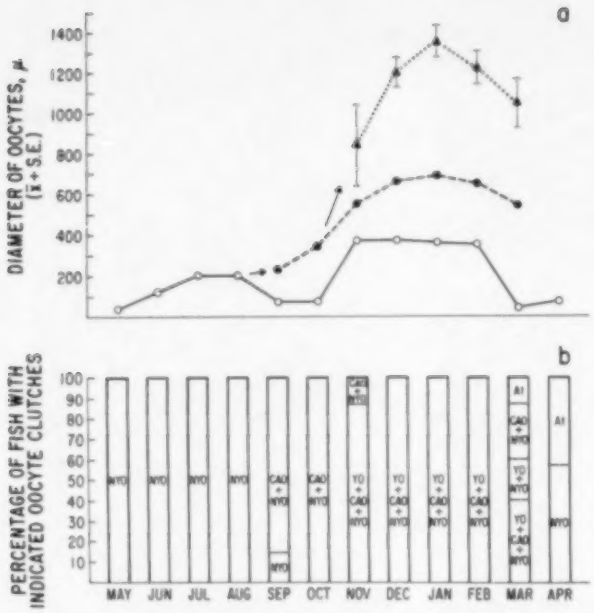

Fig. 2. Seasonal variation of oocyte (i.e., follicle) diameters and ovarian clutch composition in *Leptocottus armatus*. Data for July and August are extrapolations. Sample size in each month is 12–15 except for October where $N = 8$. (a) Mean diameter of oocytes in distinct clutches. (▲·····▲: clutch with largest diameters, i.e., leading clutch; ●– – –●: second clutch or clutch with intermediate diameters; ○——○: clutch with smallest diameters). Arrows indicate points where recruitment occurs. (b) Seasonal variation in the clutch composition of ovaries. Percentage of females in the sample with the indicated clutch or clutches is plotted as a function of month of collection. (YO—clutch of oocytes with yolk inclusions; CAO—clutch of oocyte containing cortical alveoli; NYO—clutch of oocytes without cortical alveoli; At—atretic follicles predominate in the ovary.)

only non-yolky oocytes, the diameters of which never exceed 725 μm. In *Leptocottus* 2–3 clutches (size classes) of oocytes were clearly identified in the ovary of an individual fish collected September through March (Fig. 2). Within a given clutch, oocyte diameters were remarkably uniform. Uniformity of size was greatest in the leading clutch (size group with largest diameters), groups of non-yolky oocytes being more heterogenous in diameter. When 3 clutches existed in an individual ovary 3 developmental stages were consistently represented: a clutch of vitellogenic oocytes (Figs 1B and C) with diameters in the 700–1470 μm range (in an individual ovary the vitellogenic clutch consisted of oocytes of a uniform diameter), a clutch of oocytes in the cortical alveoli stage (Fig. 1A) with diameters in the 250–700 μm range (in an individual ovary the cortical alveoli clutch possesses oocytes of a relatively uniform diameter), and a clutch of non-yolky oocytes with diameters of less than 210 μm which contained non-yolky oocytes. Oocyte development in *Leptocottus* is, therefore, group-synchronous. Recruitment of a clutch of oocytes into vitellogenesis occurred when the diameter of oocytes was approximately 700 μm. Normally there was only one clutch of oocytes undergoing yolk accumulation (vitellogenesis); commonly 1 clutch was "arrested" in a stage where there had been extensive accumulation of cortical alveoli.

Regardless of female standard length or body weight, the diameter of ovulated eggs in *Leptocottus* was 1600–1620 μm. Near the time of oocyte maturation and subsequent ovulation, yolk proteins coalesced to yield a rather solid mass of fluid yolk. Thus ovulated eggs were more translucent than intrafollicular oocytes. Ovulated eggs contained lipid droplets (generally 3–7) just under the membrane on one pole; on the opposite pole of the egg was a less translucent region.

In *Leptocottus* ovulation and oviposition were not coupled. Ovulated eggs, awaiting oviposition, filled the ventral 85–95% of an ovary; clutches of developing oocytes occupying an elipsoidal area in the mediodorsal portion of the ovary. When ovulated eggs were present in the ovarian lumen, the diameter of oocytes in the next clutch never exceeded 925 μm.

Figure 2 illustrates the seasonal changes in the clutch composition of *Leptocottus* ovaries and in the diameters of oocytes constituting these clutches. From May through August the ovaries of all fish sampled contained only non-yolky oocytes. During September and October a majority of the fish sampled possessed ovaries containing 2 clutches, a clutch of oocytes in the cortical alveoli stage and another more heterogenous clutch of non-yolky oocytes (Fig. 2b). From November through February a majority of the fish sampled revealed 3 or 4 clutches. During this period the second and third clutches were generally in the cortical alveoli stage and a fourth group of

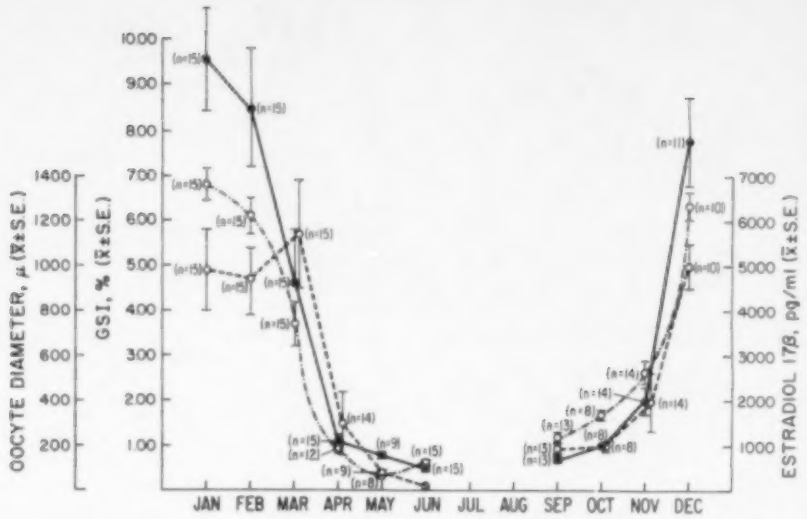

Fig. 3. Seasonal variation in diameter of oocytes (i.e., follicles), ovarian gonosomatic index (GSI) and serum estradiol-17β levels in *Leptocottus armatus*. Oocyte diameters were measured in the leading clutch only, regardless of the number of clutches in that ovary (i.e., average oocyte diameter in an ovary was not used in calculating these means). ○—·—○: diameter of oocytes; ○-----○: estradiol levels; ●——●: ovarian GSI.

non-yolky oocytes with diameters of 70 μm or less frequently could be detected. In the January (1981) sample, 47% of the females sampled contained large quantities of ovulated eggs in the ovary; size of oocytes in the leading clutch also peaked during this month (Fig. 2a). Ovulated eggs were noted in the ovaries of 8% and 20% of the fish sampled in December and February, respectively. These data suggest that spawning in the San Pablo Bay population of *Leptocottus* occurs during the winter months of December, January and February.

A majority of our data were collected during 1981. Samples of *Leptocottus* were also collected in January and March of 1982 to assess similarities in ovarian development. Of the 18 fish sampled in January 1982, all had at least 3 distinct clutches of oocytes in the ovaries and 50% of the fish had ovulated eggs in the ovarian cavity. The GSI for this sample was 13.66 ± 2.47 ($\bar{X} \pm$ SE) and the diameter of oocytes in the leading clutch was $1338 \pm 82\,\mu$m ($\bar{X} \pm$ SE). Ovarian GSI in the March 1982 sample was 1.92 ± 0.50 ($\bar{X} \pm$ SE, $N = 16$), and the diameter of oocytes in the leading clutch was $595 \pm 171\,\mu$m ($X \pm$ SE, $N = 8$). Atretic follicles predominated in the ovaries of 47% ($N = 16$) of the females sampled in March 1982. Since serum protein-bound phosphorus levels were similar in the March samples of 1981 and 1982 (Fig. 4), ovarian development in the 1982 samples was equivalent to the counterparts of 1981.

Given convincing evidence that estradiol-17β promotes hepatic synthesis of the yolk precursor, vitellogenin (deVlaming *et al.*, 1980), one of our aims was to examine the relationship of seasonal variations in oocyte development and serum estradiol-17β levels.

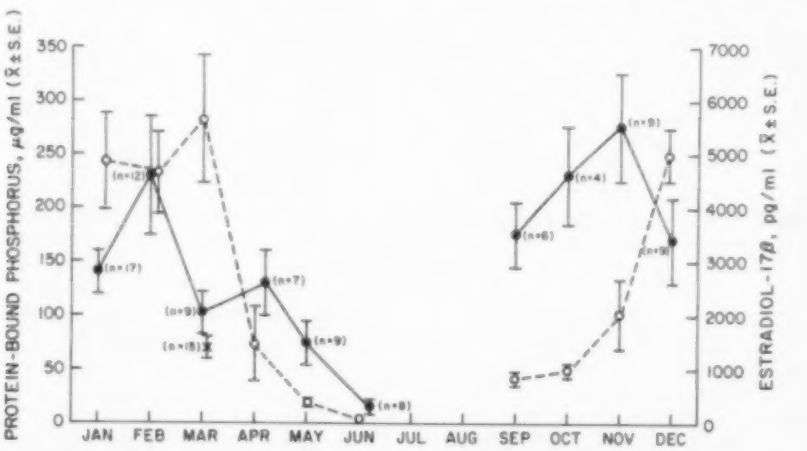

Fig. 4. Seasonal variation of serum protein-bound phosphorus (i.e., vitellogenin) and estradiol-17β levels in *Leptocuttus armatus*. ●——●: protein-bound phosphorus; ○-----○: estradiol. ×—protein-bound phosphorus levels in sample taken during March 1982.

Annual changes in serum estradiol levels are summarized in Figs 3 and 4. During the months of December, January, February and March serum estrogen levels were elevated (>4000 pg/ml). A sharp decline in serum estrogen levels occurred in April (compared to March levels). Serum estradiol-17β levels remained low from May through October (Fig. 3). Yolky oocytes did not occur in the ovaries of fish collected in August or September, but were evident in the ovaries of females sampled in November. This is notable since serum estradiol-17β levels began to increase (i.e., were higher) during November compared to the previous 6 months. In females where atretic follicles predominated in the ovaries estradiol levels never exceeded 525 pg/ml. Moreover, seasonal variation in mean serum estradiol levels showed a significant ($P < 0.01$) correlation with the annual changes of the mean oocyte diameter in the leading clutch ($r = 0.928$; $N = 10$) and with the GSI ($r = 0.894$; $N = 10$). Within the monthly samples, however, serum estradiol levels were correlated with oocyte size or GSI in the November collection only.

With data collected throughout the year (i.e., all females collected), serum estradiol-17β levels were correlated with both oocyte size and ovarian GSI. Worth clarifying, however, is that on a statistical basis only 16% of the variation in estrogen levels could be attributed to GSI changes. In the 55 females collected during the spawning season serum estrogen levels were not correlated with ovarian GSI nor oocyte size.

Since estradiol induces hepatic synthesis of the yolk precursor in teleostean fishes, another objective of this study was to determine seasonal variations of serum protein-bound phosphorus (indicator of vitellogenin). We also examined the relationship of this measurement to oocyte development and serum estrogen levels. Shown in Fig. 4 is the annual change of serum protein-bound phosphorus. Serum protein-bound phosphorus increased in parallel with oocyte size and serum estradiol levels from September through November (Fig. 4). Oocyte yolk accumulation was not obvious until November (Fig. 2) even though serum vitellogenin levels were high during September and October. With the onset of ovulation during December, serum vitellogenin levels declined (Fig. 4). Serum vitellogenin levels during January (month in which the greatest number of fish with ovulated eggs were sampled) were similar (i.e., low compared to November and February samples) to those of the December sample, even though serum estradiol levels were high during both December and January. Average ovarian size began to decrease during February (Fig. 3), yet serum estradiol and protein-bound phosphorus remained high (Fig. 4). Although serum vitellogenin levels and ovarian size declined in March with the onset of gondal regression, serum estrogen levels remained high. From April through June serum protein-bound phosphorus and estradiol levels decreased in parallel (Fig. 4). The seasonal variations (i.e., monthly means) of serum estradiol and protein-bound phosphorus were not significantly correlated, nor was there a significant correlation when an analysis was performed with data collected from females collected throughout the year.

Studies with some teleostean species indicate that alterations in serum protein levels reflect changes in serum vitellogenin levels and in reproductive state. Although we noted a seasonal variation of serum protein concentration in female *Leptocottus*, this parameter was not correlated with serum protein-bound phosphorus. A pronounced increase of serum protein occurred with the onset of oocyte maturation and ovulation in December (Table 2). During the month (January) of peak ovulation, serum protein declined to approximately the level noted during the non-breeding season. Serum protein levels were again elevated in February, and remained high during March and April. By May, when atresia was complete, serum protein levels had declined and were at a low point in the June sample. Calculations showed that serum protein levels were not correlated with serum estradiol levels nor with ovarian size in *Leptocottus*.

Lipid forms a component of vitellogenin and is incorporated into growing oocytes, especially in marine fishes. That estrogen is involved in mobilization of lipid reserves during ovarian recrudescence has been proposed. Visceral lipid reserves and plasma total lipids were assayed in this study. One of our objectives was to examine the seasonal variation in these lipid parameters and to relate these measurements to ovarian development as well as serum estrogen levels. A pronounced seasonal variation of plasma total lipid levels occurred in female *Leptocottus* (Fig. 5). Plasma total lipid levels were highest just prior to the onset of vitellogenesis (i.e., October and September); levels remained high during vitellogenesis and throughout the breeding season (November through February—Fig. 5). A slow decline in mean serum total lipid levels was evident during the spawning period (December through February). Serum total lipid levels remained relatively high during the phase of ovarian regression (March and April), but declined to very low levels in May and June when the ovaries were regressed (Fig. 5). The annual variation of mean serum total lipids was not significantly correlated with the mean serum estrogen titer, mean oocyte diameter, or mean ovarian GSI.

Visceral lipid reserves in *Leptocottus* varied markedly throughout the year (Fig. 5). Following the spawning season (e.g., April) visceral lipid reserves began to increase and continued to increase throughout the spring and summer. As ovarian enlargement commenced during October and November visceral lipid reserves began to decline (Fig. 5). Visceral lipid reserves were lowest during the spawning period (December through March). The annual variation (i.e., monthly means) of visceral lipid reserves was inversely correlated with seasonal GSI ($r = -0.69$; $P < 0.05$; $N = 10$) and estradiol ($r = -0.77$; $P < 0.01$; $N = 10$) changes. A statistically significant correlation between visceral lipid reserves and serum total lipids was not evident.

DISCUSSION

Some females in the population of staghorn sculpins (*Leptocottus armatus*) occurring in San Pablo Bay, California are capable of spawning from mid-December to mid-March. It is during these months

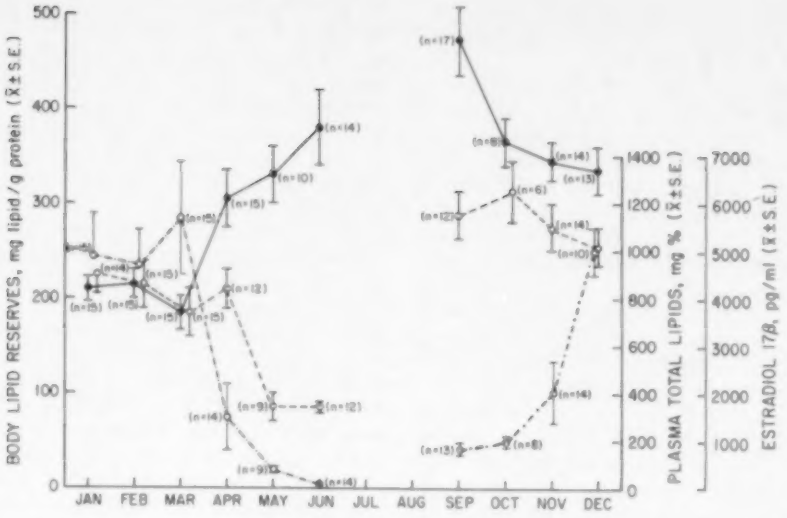
BODY LIPID RESERVES, mg lipid/g protein (X̄±S.E.)
PLASMA TOTAL LIPIDS, mg % (X̄±S.E.)
ESTRADIOL 17β, pg/ml (X̄±S.E.)
JAN FEB MAR APR MAY JUN JUL AUG SEP OCT NOV DEC

Fig. 5. Seasonal variation of serum total lipid, body lipid reserves (i.e., visceral lipid stores) and estradiol-17β levels in *Leptocottus armatus*. ○-----○: total serum lipids; ○——○: estradiol ●——●: body lipid.

that ovarian weights and yolky oocyte diameters (Fig. 3) were at their maxima. Basically an identical spawning period has been predicted (Tasto, 1975) for a population of *L. armatus* occurring in Anaheim Bay, California (approximately 4° latitude south of San Pablo Bay). Jones (1962) concluded that spawning in the San Francisco Bay (to which San Pablo Bay is broadly connected) population of *Leptocottus* occurs from October through March with a peak during January–February. We predict a spawning peak during January based on the number of females sampled containing ovulated eggs in the ovarian lumen. Since most marine and euryhaline cottid fishes studied spawn during winter (Breder and Rosen, 1966), *Leptocottus* is not atypical. Ovarian regression in the San Pablo Bay population of *Leptocottus* was initiated in mid to late March and completed by May. From May through August only non-yolky oocytes occurred in the ovaries. Ovarian recrudescence was initiated during September; this recrudescence was marked by the enlargmenet of oocytes and formation of cortical alveoli (Figs 1A, 2b). During late October or November growing oocytes begin to accumulate yolk.

Oocyte development in *Leptocottus* is group-synchronous as 2–4 distinct size classes (clutches) of oocytes were recognized in the ovary of an individual female preceding and during the spawning period (Fig. 2). One of these clutches contained oocytes in the process of vitellogenesis or post-vitellogenesis, a second (and sometimes a third) clutch contained oocytes "arrested" in the cortical alveoli stage, and a third (or sometimes a fourth) clutch contained non-yolky oocytes in the primary growth phase. As in several other species (e.g., deVlaming, 1983), recruitment of a clutch from the cortical alveoli stage into vitellogenesis normally did not occur in *Leptocottus* until maturation was initiated in the leading clutch. Since the yolk accumulation process is gonadotropin dependent (Wiegand, 1982), an intriguing question, of interest to endocrinologists, concerns the apparent refractoriness (in terms of vitellogenesis) to tonic gondotropin levels of the second clutch, as compared to the leading clutch. Oocyte maturation and ovulation are initiated by a dramatic increase of plasma gonadotropin levels in the goldfish (Stacey *et al.*, 1979). Should there be an equivalent "ovulatory surge" of gonadotropin in *Leptocottus*, it might also function to recruit the clutch of oocytes in the cortical alveoli stage into vitellogenesis. Support for this contention is provided by Wallace and Selman (1979) in work with the four-spined stickleback, *Apeltes quadracus*. They reported that injections of human chorionic gonadotropin recruited oocytes into the yolk accumulation phase even when a vitellogenic clutch was existent.

Although three distinct clutches of oocytes could be recognized in the ovary of a female *Leptocottus* preceding and during the breeding season, we have no clear evidence of more than one oviposition per female in an annual spawning season. Moreover, Jones (1962) claimed that a *Leptocottus* female spawns but once per year. We noted extensive reabsorption of yolky oocytes during March and April, indicating that clutches 2 and 3 were not spawned. Group-synchronous oocyte development with the spawning of more than 1 clutch of oocytes per season has been reported for other cottid species (DeMartini, 1978; Fox, 1978; Goldberg, 1980; deVlaming *et al.*, 1982). In concordance with the *Leptocottus* data, shorthorn sculpin, *Myoxocephalus scorpius*, females spawn but once per year, yet reveal 3 distinct oocyte clutches (Ennis, 1970). Development of clutches which are not spawned seems nonadaptive from an energetics standpoint. Oocyte development in the San Pablo Bay population of *L. armatus* is not synchronized (i.e., females in various phases of vitellogenesis were collected between November and March). A prudent assumption seems to be that certain environmental conditions (perhaps low water temperatures) are permissive for ovarian re-

crudescence, yet vitellogenesis and ovulation do not seem to be precisely synchronized by environmental factors.

In *Leptocottus* serum estradiol-17β levels began to increase (compared to the previous 6 months) during November coincident with the appearance of yolky oocytes in the ovary. This is consistent with the role of estrogen in promoting hepatic synthesis of the yolk precursor (see below). In contrast to most other teleostean species examined, serum estradiol titers in *Leptocottus* were highest during the period of ovulation and spawning (December through March). Estrogen levels decline sharply prior to ovulation or spawning in *Cyprinus carpio* (Eleftheriou *et al.*, 1968), *Pleuronectes platessa* (Wingfield and Grimm, 1977), *Salmo gairdneri* (Lambert *et al.*, 1978), and *Salvelinus leucomaenis* (Kagawa *et al.*, 1981). Should estrogen exert only a negative feedback control on gonadotropin secretion in this cottid species, one would predict relatively low gonadotropin levels during the period of oocyte maturation and ovulation. Moreover, provocative questions concern the potential role of a gonadotropin surge in oocyte maturation and ovulation in *Leptocottus*, as well as, a positive feedback effect of estrogen on gonadotropin secretion. Should there be more than one gonadotropin (e.g., Idler, 1982) in *Leptocottus*, estrogens could effect their secretion differentially.

Seasonal variations of serum estradiol levels generally paralleled changes in stage of oocyte development and ovarian GSI (Fig. 3). Nonetheless, serum estradiol titers were not significantly correlated with oocyte size nor GSI during the months of spawning (December through February) or within but one of the monthly samples. Furthermore, when data from all females collected throughout the year were used the correlation coefficient between ovarian size and serum estradiol levels was relatively low. Thus serum estrogen levels in this sculpin were not necessarily dependent on ovarian mass, and certainly, ovarian size cannot be used as an index for predicting estradiol levels. These findings with *Leptocottus* are reminescent of those reported for the bluefish, *Pomatomus saltator*. Although plasma estrogen levels in this pomatomid species vary seasonally, estradiol titers and ovarian GSI are not significantly correlated (MacGregor and Finucane, 1981). Wingfield and Grimm (1977) reported a parallel increase of plasma estradiol levels and ovarian GSI in *Pleuronectes platessa* with estrogen peaking 1$\frac{1}{2}$ months prior to spawning. A significant correlation between plasma estradiol titers and ovarian GSI or between the seasonal variation in mean GSI and mean plasma estrogen levels has been noted in a few teleostean species (*Cyprinus carpio*—Eleftheriou *et al.*, 1968; *Tilapia aurea*—Yaron *et al.*, 1977; *Mugil cephalus*—Dindo and MacGregor, 1981; *Scomberomous cavalla*—MacGregor and Finucane, 1981). Seasonal variations of plasma estrogen have been most frequently determined in salmonid fishes. Both estradiol-17β and estrone peak during the yolk accumulation phase of the ovarian cycle and decline during the spawning period in *Salmo gairdneri* (van Bohemen and Lambert, 1981). Generally, high plasma levels of estradiol are associated with vitellogenesis, whereas low titers occur during the spawning period among salmonids (Cedard *et al.*, 1961; Breton *et al.*, 1975; Fostier *et al.*, 1978; Whitehead *et al.*, 1978a,b; Lambert *et al.*, 1978; Kagawa *et al.*, 1981). Plasma estrogen titers are more likely to be correlated with ovarian GSI, and also be low during the spawning season, among teleostean species having relatively synchronous ovarian development at the population level. That estrogen levels are more related to favorable environmental and endocrine conditions than to ovarian mass seems probable.

Estrogenic induction of a female-specific serum protein, the yolk precursor vitellogenin, in males or non-vitellogenic females has been demonstrated in several teleostean species (Ho and Vanstone, 1961; Plack *et al.*, 1971; Aida *et al.*, 1973; Campbell and Idler, 1976; Emmersen and Petersen, 1976; deVlaming *et al.*, 1977a, 1980; Hara and Hirai, 1978; Idler and Campbell, 1980; Sundararaj and Nath, 1981). Nonetheless, few investigators have analyzed the relationship of plasma vitellogenin levels to oocyte development or to plasma estradiol-17β titers. We examined these relationships in *Leptocottus armatus* using protein-bound phosphorus as an indicator of serum vitellogenin levels. Protein-bound phosphorus is accredited as an accurate indicator of vitellogenin levels among teleosts (e.g., deVlaming *et al.*, 1980; Sundararaj and Nath, 1981). Should the degree of vitellogenin phosphorylation change during the season, this index might be somewhat biased. Indeed, van Boheman *et al.* (1981) implied that the *Salmo gairdneri* yolk precursor is more phosphorylated at the end of the vitellogenic phase than at the beginning.

From September through November serum protein-bound phosphorus increased coincident with oocyte size and estradiol levels (Fig. 4); histological evidence of oocyte yolk accumulation was not manifested until November (Fig. 2). Serum vitellogenin levels in *Leptocottus* were lower during December and January than in the November and February samples. The single point samples (i.e., samples taken on 1 day) may not be reflective of the entire month, yet the lowered serum levels possibly disclose a high rate of yolk precursor uptake during this period. Both estradiol and vitellogenin levels were high during February (towards the end of the spawning phase). The elevated vitellogenin levels in February compared to December/January (early spawning phase) could be indicative of a decline in uptake by oocytes. Although estradiol titers were very high during March, serum vitellogenin levels were low. This observation remains a mystery, especially since this month marked the onset of ovarian regression. Serum vitellogenin and estradiol levels declined in concert with ovarian weight from March through June. Although it is tempting to propose that the liver in *Leptocottus* is more responsive (with regards to vitellogenin synthesis) to estrogen during the late summer and autumn than during late winter and spring (see Fig. 4), serum levels of the yolk precursor depend, not just on estrogen, but also on rates of synthesis, degradation, and uptake. That neither GSI nor estradiol titers were significantly correlated with serum protein-bound phosphorus levels in *Leptocottus* also may be related to these dynamics.

Among some teleostean species protein-bound

phosphorus concentration rises in parallel with GSI (and oocyte yolk accumulation) during ovarian recrudescence, levels beginning to decline prior to spawning (*Esox lucius*—Goedmakers and Verboom, 1974; *Platichthys flesus*—Petersen and Emmersen, 1977; *Salmo trutta*—Crim and Idler, 1978). In the rainbow trout (*Salmo gairdneri*) plasma vitellogenin levels peak just before oocyte maturation and ovulation (i.e., are highest during the yolk accumulation phase), but, as in *Leptocottus*, remain relatively high during the spawning period (Whitehead *et al.*, 1978b; van Bohemen and Lambert, 1981). On a seasonal basis plasma estrogen titers peak 1 month prior to vitellogenin levels in *S. gairdneri* (van Bohemen and Lambert, 1981). These data contrast to our findings with *Leptocottus* where serum vitellogenin levels peak before estradiol levels (Fig. 4). In *S. gairdneri* (van Bohemen and Lambert, 1981), *Scyliorhinus canicula* (Craik, 1979) and *Leptocottus* serum estradiol and vitellogenin levels are not significantly correlated. Generalizations concerning the correlation between serum estrogen and vitellogenin levels must, therefore, be made with considerable caution. Furthermore, among teleosts the relationship between serum vitellogenin levels and the ovarian cycle show intraspecific differences.

Although serum protein levels in female *Leptocottus* varied seasonally, the changes did not parallel variations in serum vitellogenin. Of particular note were the relatively low levels of serum protein during the ovarian recrudescence phase (September–November). Serum levels in *Leptocottus* were elevated during the spawning (with the exception of January) and ovarian regression phases. Although some investigators have reported that plasma protein levels are comparatively high during the vitellogenic phase and can be used as an index of vitellogenin levels (*Carassius auratus*—Bailey, 1957; *Anguilla anguilla*—Lewander *et al.*, 1974; *Cyprinus carpio* and *Vagrus bayad*—Hilmy *et al.*, 1978), we recommend that this relationship be confirmed on a species by species basis. Lending credence to this reservation are the data of van Bohemen *et al.* (1981). According to these investigators plasma protein and vitellogenin concentrations are not correlated in *S. gairdneri*. In other work with rainbow trout Campbell and Idler (1980) demonstrated that estradiol treatment elevates vitellogenin levels at the expense of other plasma proteins. Treament of several teleostean species with estrogen results in an elevation of plasma protein levels (Bailey, 1957; Yaron *et al.*, 1977, 1980; Terkatin–Shimony and Yaron, 1978; deVlaming *et al.*, 1979; Medda *et al.*, 1980; van Bohemen *et al.*, 1982). Generally, however, pharmacological doses of the sex steroid have been administered.

According to Shatunovsky (1971) and Shchepkin (1971) the stored lipids most extensively mobilized during ovarian recrudescence among teleosts are triglycerides. Triglyceride incorporation into growing oocytes of *Platichthys flesus* has been reported (Lapin, 1973). Dave *et al.* (1974) suggest that triglycerides constitute the greatest percentage of lipids in the oocytes of *Anguilla anguilla*. On the contrary, other investigators (Shatunovsky, 1971; Lapin, 1971; Lizenko *et al.*, 1973) contend that phospholipids constitute up to 60%, cholesterol up to 35%, and triglycerides up to 35% of teleostean ovarian lipids. Apparent discrepancies could relate to variations in stage of ovarian development or species variability. In any case, evidence which shows that lipids are mobilized during ovarian development is mounting. Visceral lipid stores began to accumulate with the onset of ovarian regression and continued to increase until the onset of ovarian recrudescence in *Leptocottus* (Fig. 5). With the initiation of ovarian recrudescence visceral lipid reserves began to decline and continued to decrease through the vitellogenic and spawning phases. The significant inverse correlation between visceral lipid reserves and ovarian GSI in *Leptocottus* suggests a functional relationship between lipid mobilization and reproduction. That lipids stored in these visceral deposits are mobilized for deposition in developing oocytes, vitellogenin synthesis and/or metabolism seems feasible. Such a hypothesis is supported by the observation that serum lipid levels were high during ovarian recrudescence, remained elevated during the spawning season and until ovarian regression was complete (Fig. 5). The low serum lipid levels during late spring and early summer coincided with lipid deposition (Fig. 5).

Previous reports of total lipid levels in fish plasma failed in mention that the sulphophosphovanallin spectrophotometric procedure depends on the presence of unsaturated fatty acids in the lipids. Should the ratio of unsaturated to saturated fatty acids in lipids change with season, this assay could yield biased data. Elevated temperatures generally result in an increased production of saturated fatty acids (e.g., Cowey and Sargent, 1979), so the apparent decline of serum total lipid levels in *Leptocottus* during the summer months may be due, in part, to an increase of saturated fatty acids. Regardless of the seasonal change in the fatty acid composition of lipids among teleosts, polyunsaturated fatty acids predominate in all seasons.

As in *Leptocottus*, visceral or total body lipid reserves are depleted during or just prior to the vitellogenic phase in other teleostean species (MacKinnon, 1972; deVlaming, 1975; Delahunty and deVlaming, 1980). Also consistent with our data on *Leptocottus* , serum lipids are high during ovarian recrudescence and the breeding season in *Mugil cephalus* (Dindo and MacGregor, 1981). After an initial decline at the initiation of the oocyte yolk accumulation phase, plasma total lipid levels in *Platicthys flesus* increase to the onset of the spawning period; plasma lipid levels decrease to low levels through the spawning season (Petersen and Emmersen, 1977). Plasma phospholipid levels in this flounder increase through the oocyte secondary growth phase and decline through the spawning period (Petersen and Emmersen, 1977). In the cod, *Gadus morhua*, plasma lipid levels increase in connection with ovarian growth, decreasing during the spawning period (Plack and Woodhead, 1966). Since fat stores, as well as plasma lipid levels, can relate to food availability, food composition, feeding behavior, temperature and other factors, it is not our intention to imply that all changes in these lipid parameters are related to reproduction. Worthy of note is a study with the

plaice, *Pleuronectes platessa*, where 50% of the increase in ovarian size occurs when females are not feeding. Of the decline in body lipid reserves during this time only 14% is utilized for egg production, 50% being metabolized for energy (Dawson and Grimm, 1980).

Visceral lipid stores in female *Leptocottus* began to increase during the post-spawning period as estrogen levels declined (Fig. 5). During ovarian recrudescence, viscerel lipid reserves were depleted as estrogen levels increased. In fact, a significant inverse correlation between visceral lipid reserves and serum estrogen levels was calculated. There is a potential, therefore, of a functional relationship between lipid mobilization and estradiol levels. Since we did not separate the liver from the other visceral organs, it is not possible for us to predict whether lipid in the mesentary deposits was first channeled to the liver. Nor can we conclude that liver lipid content varies in parallel with lipid content of the total visceral mass. Estradiol administration has been shown to elevate liver lipid levels among teleostean species (e.g., Takashima *et al.*, 1972; deVlaming *et al.*, 1977a,b; Emmersen *et al.*, 1979). Plasma total lipid (Takashima *et al.*, 1972; deVlaming *et al.*, 1977a; Wiegand and Peter, 1980), phospholipid (Bailey, 1957; Urist and Scheide, 1961; Plack and Pritchard, 1968; Sand *et al.*, 1980; Wiegand and Peter, 1980), and cholesterol (deVlaming *et al.*, 1979; Wiegand and Peter, 1980) levels increase following estrogen treatment in several teleostean species. Taken together, these data support the contention that estradiol mobilizes lipids, some of these being sequestered by the liver (for vitellogenin synthesis or metabolic modification?).

Combined, our data on the ovarian cycle, as well as associated endocrine and metabolic events of *Leptocottus* form a basis for initiating more specific experimental studies. In addition to providing new perspectives on aspects of piscine reproduction adaptations, our findings further illustrate that generalizations concerning teleostean reproductive physiology and endocrinology are not prudent.

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INVOLVEMENT OF THE GILL CHLORIDE CELLS OF TROUT UNDER ACUTE NITRITE INTOXICATION

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Abstract—1. In the gill of trout (*Salmo gairdneri* Rich.) treated with 450 μg N–NO_2/l for 72 hr, ultrastructural changes are seen especially in chloride cells, probably due to an accelerated turnover.

2. Despite the marked inhibition exerted by nitrite on gill carbonic anhydrase activity, blood Cl^- content of treated trout is not altered.

3. The hypertrophia observed in some gill chloride cells could be a hyperactive response attempting to maintain physiological Cl^- levels in the presence of NO_2^- and lowered HCO_3^- production.

INTRODUCTION

Nitrite is a serious toxicant to freshwater fish even when it is present in the environment in a relatively low concentration, because nitrite rapidly moves from the water to inside the organism, so reaching surprisingly high concentrations in blood and tissues (Margiocco *et al.*, 1983). Both nitrite toxicity and blood accumulation are known to be lowered by environmental chloride and by inhibitors of Cl^- uptake (Bath and Eddy, 1980). For this reason it has been suggested that NO_2^- competes with Cl^- in the active chloride uptake by the chloride cells in the gill epithelium of freshwater fish (Bath and Eddy, 1980). This hypothesis explains the strong bioaccumulation characteristic of nitrite because it is presumed that nitrite is involved in a pump mechanism arranged for a physiological ion uptake.

In order to study the concentrating mechanism for NO_2^- in freshwater fish, we studied the changes of gill chloride cells in trout exposed *in vivo* to high nitrite concentration.

Besides morphological changes, functional alterations are expected to occur particularly in the activity of carbonic anhydrase, a key-enzyme in Cl^- flux across cell membrane. Current opinion about the mechanism concerning branchial chloride absorption presumes an HCO_3^-/Cl^- exchange mechanism.

The effects of nitrite on carbonic anhydrase activity were studied *in vitro* by means of experiments in which the nitrite concentration observed *in vivo* was simulated. The nitrite accumulated in gills under *in vivo* exposure is markedly diluted during enzyme extraction procedures. This must be avoided, because the action of several anions on carbonic anhydrase activity is reversed when they are removed from the medium.

MATERIALS AND METHODS

(*A*) In vivo *treatments*

Salmo gairdneri specimens, about 18–20 cm in length, were acclimatized for 15 days in 600 l capacity tanks supplied with a continual flow of water from Lake Maggiore, at the Istituto Italiano di Idrobiologia (Pallanza).

Water characteristics were as follows: temperature 12°C; conductivity = 107 μS (18°C); total ammonia (μg N/l) = 18; NO_2^- (μg N/l) = 6; NO_3^- (μg N/l) = 445; PO_4^- (μg P/l) = 3; total P (μg P/l) = 12; Cl^- (mg/l) = 2; hardness (mg/l $CaCO_3$) = 66; pH = 7.4.

At the beginning of the experiment, $NaNO_2$ was added to the tanks (except for the one containing control trout) so as to attain a concentration of 450 μg N/l of nitrite–nitrogen (NO_2–N). Such a concentration was maintained constant during the experiment by a continual flow of nitrite-added lake water.

During the time of the experiment, fish were fed twice a day and maintained in normal photoperiodic conditions. After 72 hr of exposure to the toxicant, the survived fish were killed by decapitation, and their gills were removed.

Ultrastructural techniques. For the ultrathin section technique, gills were prefixed at room temperature in a 2.5% solution of gluteraldehyde buffered at pH 7.2–7.4 with phosphate for 2–4 hr. During prefixation the livers were cut into small pieces which, after washing in the buffer, were postfixed in 1% osmium tetroxide, buffered as above, at 4°C for 1 hr. After fixation the tissues were dehydrated in graded acetone and imbedded in Durcupan. Ultrathin and thin sections were cut with a diamond knife. The former were stained with uranyl acetate and lead citrate (Reynolds, 1963). The latter were stained with methylene blue. The observations were performed using Zeiss 9M electron microscope.

(*B*) In vitro *treatments*

Gill filaments were trimmed from supporting arches of killed fish. Filaments, minced and washed in HEPES 15 mM, were homogenized in the same buffer and centrifuged at 3000 *g* for 10 min.

Carbonic anhydrase activity was determined on the supernatant measuring the time required for pH to drop from 8.3 to 6.8 during enzymic hydratation of CO_2. Assay was carried out electrometrically according to Johnston and Jungreis (1979). All the assay procedures were carried out at 2°C. The activity unit was calculated according to the following expression:

$$\frac{T_0 - T}{T} \Big/ (\text{mg prot.})$$

where T_0 and T are the reaction times for the uncatalyzed and catalyzed reaction, respectively (Waygood, 1955).

RESULTS

(*A*) *Ultrastructural studies*

Control trout. According to Morgan and Tovell (1973), in *Salmo gairdneri* chloride cells are distrib-

uted uniformly in the lamellar epithelium (Fig. 1(a)). In the lamellae of control trout, whose central axis shows wide blood spaces (Fig. 1(b)), chloride cell morphology follows the descriptions given by previous ultrastructural studies in *Salmo gairdneri* gills (Olson and Fromm, 1973; Morgan and Tovell, 1973; Kendall and Dale, 1979; Kimura and Kudo, 1979).

Chloride cells are more ovoidal in shape than epithelial cells and their surface area, partially covered by microprojections of neighbouring epithelial cells, protrudes outside with numerous microvilli (Fig. 1(c)).

Microvilli are exhibited also by epithelial cells and a fibrillar electron-opaque coat covers the microvilli of all superficial cells (Fig. 1(c)).

In the cytoplasm of chloride cells, particularly enriched in mitochondria, the agranular endoplasmic reticulum is well represented while ribosomes and elements of the granular one are randomly distributed within the cytoplasmic matrix (Fig. 1(d)).

The apical region is free in mitochondria and enriched in many micro-vesicles with strongly electron-opaque granules (Fig. 1(e)). The nucleus, large and not very electron-opaque, is located in the basal part of the cell (Fig. 1(e)).

As was pointed out by Threadgold and Houston (1964) in *S. salar*, in the control gill it is possible to observe that the shape of chloride cells is related to their location: the cells localized among the lamellae are cube-shaped (Fig. 1(c)), while the cells distributed on the lamellae are flattened (Fig. 1(e)). No apical pit was observed, as previously described in *S. gairdneri* chloride cells (Morgan and Tovell, 1973; Kendal and Dale, 1979).

Treated trout. Chloride cells localized on the secondary lamellae are very globous and protrude outside to occupy a large part of the interlamellar space (Fig. 2(a)).

The ultrastructural analysis shows the cell tendency to hypertrophia as, unlike controls, chloride cells are projected outside in the surrounding medium. These cells show a nude surface free from the epithelial coat (Fig. 2(b)) and present microvilli (Fig. 2(c)).

As a consequence of cellular body protrusion, the plasma membranes of cells located on adjacent lamellae may sometimes meet (Fig. 2(d)).

The most striking feature of the tendency to hypertrophia is seen in some chloride cells not lying within a lamella but completely projected outside the epithelium (Fig. 2(e)).

A peduncle delimited by thin extensions of epithelial elements supports these cells so that their surfaces are almost completely in contact with the external environment.

In sections meeting the nuclear zone it may be observed that while some chloride cells have cytoplasmic features very similar to those described for control gills (Fig. 2(d)), others show cytoplasmic lacunae and a decreased electron-density in the basal region (Fig. 3(a)); these features indicate the cell is undergoing degeneration.

Vacuolation may become so extensive as to lead to the formation of vacuities, while some inclusion bodies accumulate in the cytoplasm (Fig. 3(b)). In this case the cell is completely overlapped by epithelial cells which set it apart from the external environment.

Remnants of chloride cells are seen along the lamellae of some gill filaments. Owing to the decreasing number of this cell type and the reduced diameter of blood vessels (Fig. 3(c)), the lamellae of treated gills show a thinner aspect (Fig. 3(d)). No alteration in mitochondria is seen in treated gills (Fig. 3(d)), not even in degenerating cells characterized by wide vacuolation.

(*B*) *Biochemical studies*

Carbonic anhydrase activity is markedly inhibited by nitrite levels equal or higher than 10 μg $N\text{-}NO_2$/ml (Table 1(A)), that is by levels detected in the tissues of animals exposed *in vivo* to acute intoxication (Table 1(B)).

Blood chloride values do not seem to be influenced by a prolonged exposure to high nitrite levels (Table 1(B)).

DISCUSSION

In the fish gill there is a physiological turnover of chloride cells (Peek and Youson, 1979a,b) whose stage of development and level of activity may be ultrastructurally seen on the basis of their morphological characteristics (Threadgold and Houston, 1964; Peek and Youson, 1979b; Philpott and Copeland, 1963; Conte and Lin, 1967; Shirai and Utida, 1970).

Our experimental results suggest that nitrite induces an acceleration of chloride cell turnover both producing hyperactivity in some cells enhancing their degenerating rates.

The gills of treated trout show, in comparison with controls, a greater number of chloride cells in all stages of development (active, degenerating and completely degenerated cells).

In particular, fully active cells, with intact cytoplasm, often become hypertophic as to project outside: this leads to an enhancement of the total surface area and probably to a greater absorption of dilute electrolytes from the surrounding medium. Thus hypertrophy of chloride cells indicates their hyperfunctionality. Further support to this interpretation is provided by the following observation: in treated

Fig. 1. Light microscopic (a) and transmission electron (b)–(e) views of control trout gills. (a) Semithin section showing some secondary lamellae lined with chloride cells (arrows). (b) Blood corpuscles (BC) in the wide central space of a secondary lamella. (c) Chloride cell (CC) localized among the lamellae and enveloped by lateral projections of epithelial cells (EC). (d) Cytoplasm of a chloride cell containing mitochondria (M), agranular (AER) and granular endoplasmic reticulum (GER), and ribosomes (R). (e) Chloride cell, localized on the lamellae, with a large basal nucleus (N) and aboundant mitochondria (M). Electron-opaque granules (G) are present in the thin layer free of mitochondria immediately beneath the outer surface.

Figure 1

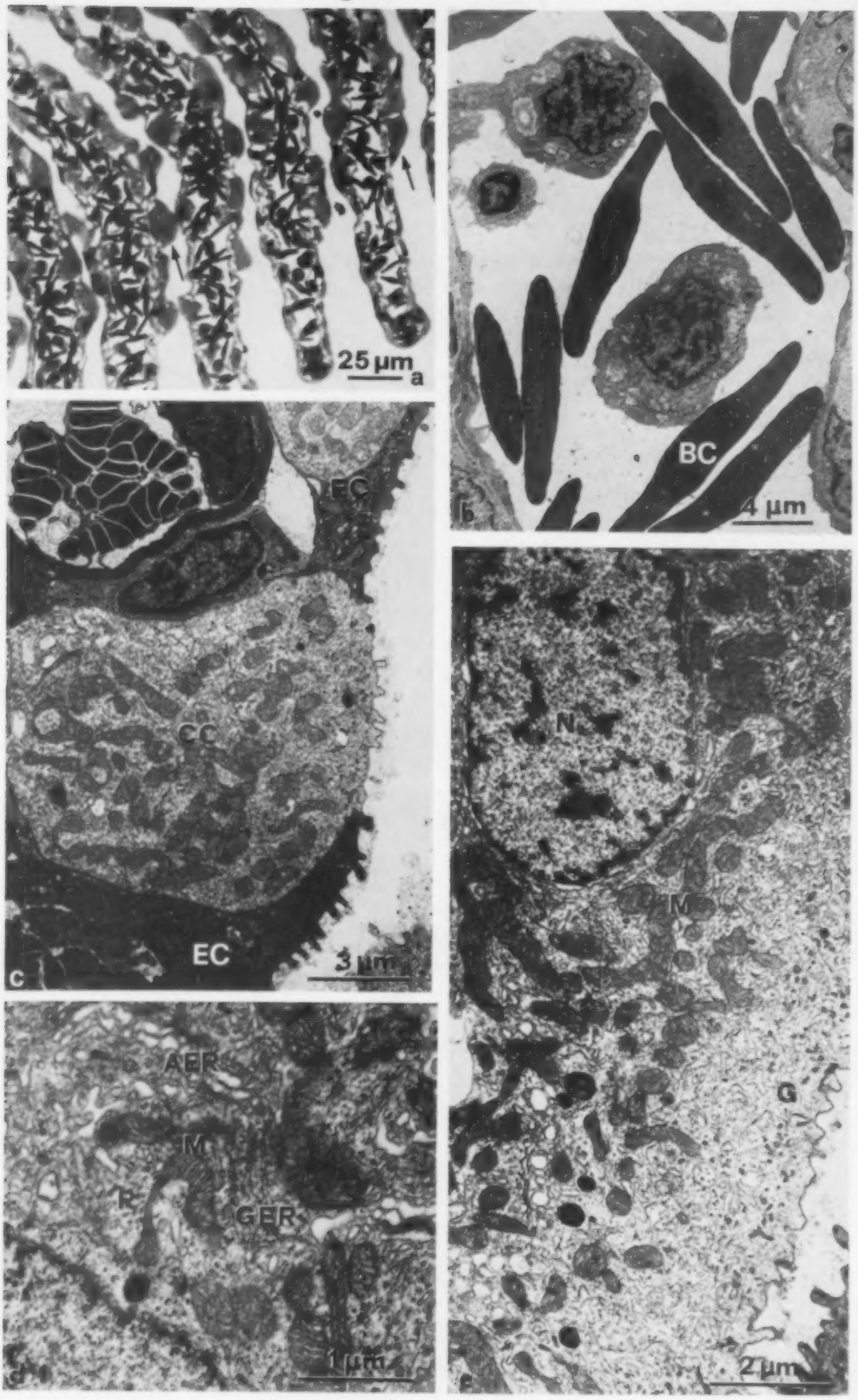


Figure 2

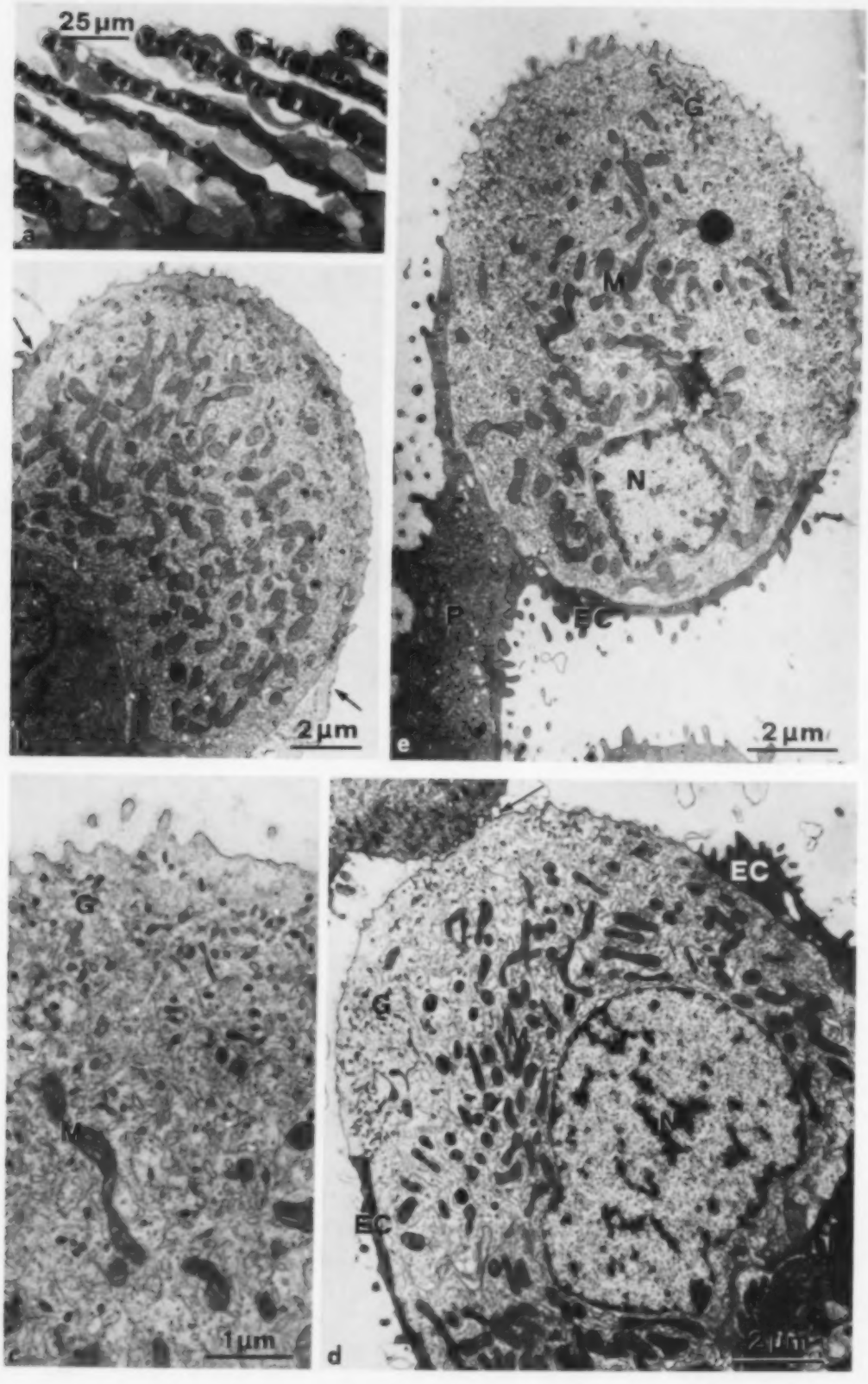


Table 1. (A) Inhibition of gill carbonic anhydrase activity (% of control) in the presence of nitrite added to the assay mixture. (B) Blood chloride concentration and nitrite bioaccumulation in tissues of trout exposed *in vivo* for 72 hr to 450 μg $N-NO_2$/litre

(A) *In vitro* experiments				
Nitrate added (μg $N-NO_2$/ml)	5	10	20	40
Carbonic anhydrase activity	74.01 ± 2.0	69.29 ± 1.5†	51.18 ± 1.2*	33.85 ± 4.0*

(B) *In vivo* experiments	Blood chloride (m eq/l)	Blood nitrite (μg $N-NO_2$/ml)	Gill nitrite (μg $N-NO_2$/g)
Control trout	89.6 ± 4	0.5 ± 0.2	0.05
Treated trout	94.5 ± 2	26.2 ± 0.9*	16.9 ± 1.3*

Data represent the mean ± SE of four samples assayed in duplicate. Statistically significant differences by Student's *t*-test.

*$P < 0.01$; †$P < 0.05$.

Data concerning tissue nitrite concentration are taken from our previous work (Margiocco *et al.*, 1983).

trout blood the Cl^- concentration remains constant in spite of a considerable uptake of nitrite that, as suggested by Bath and Eddy (1980), should compete with Cl^- for penetration.

A higher absorption of ions related to hyperactivity of chloride cells could explain both the maintainance of physiological Cl^- and the massive influx of NO_2^-.

But a further explanation is required as we also found nitrite markedly inhibits the activity of carbonic anhydrase *in vitro* even at concentrations similar to those determined in various tissues of *in vivo* treated trout. The inhibition of carbonic anhydrase should further impair the osmoregulatory function of chloride cells because of the decreased production of HCO_3^-, generally exchanged for Cl^-. Since similar impairment is not observed it cannot be ruled out that another anion exchange mechanism may occur in trout gill when carbonic anhydrase is inhibited, so maintaining the physiological uptake of Cl^-. According to this hypothesis, it is known that in trout Cl^- uptake is not impaired by acetazolamide (Kerstetter and Kirschner, 1972) despite its inhibitory effect on carbonic anhydrase. Cl^- movement across cell membrane may involve other specific ion exchanges, such as Cl^-/organic anion. Among these, Cl^-/lactate exchange described in the cornea of a toad (Bentley and Yorio, 1978) may provide an interesting model for trout gill; this model could also explain—at least partially—the lack of lactate increase in the tissue of intoxicated trout under severe hypoxia caused by nitrite-induced methemoglobinemia (Arillo *et al.*, 1984).

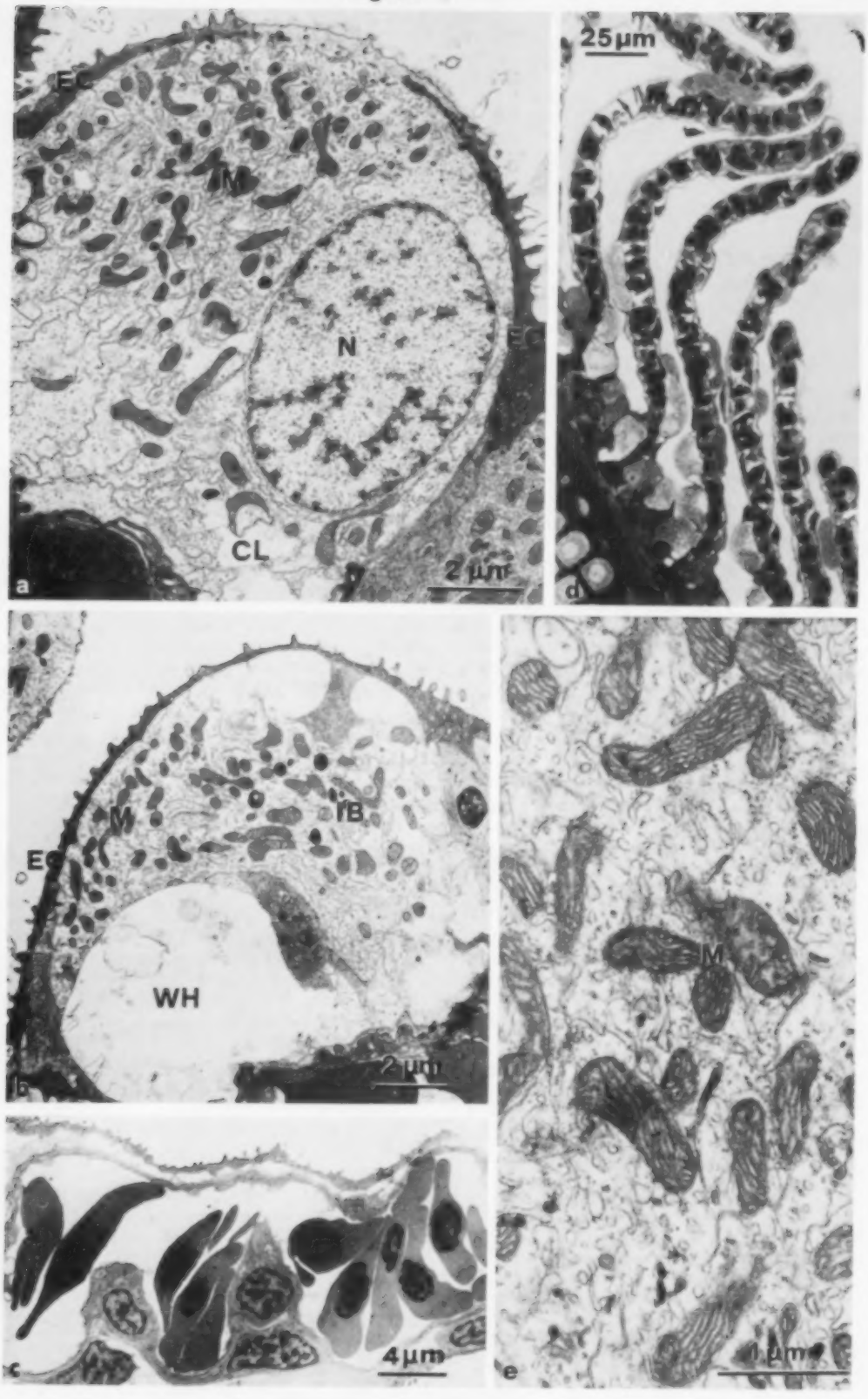
The hypertrophia observed in some gill chloride cells together with the recourse to another exchange mechanism, may be a hyperactive response to maintain physiological Cl^- levels, despite the NO_2^- competition or the decrease in HCO_3^- production. Such a response, however, seems to lead to an early cell degeneration as suggested, according to Morgan and Tovell (1973) and to Peek and Youson (1979a,b), by the presence in some cells of extensively vacuolated cytoplasm and the reduced electron density of cytoplasmic matrix.

In the gill of treated trout it is possible to observe all morphological stages between hypertrophic active cells and completely degenerate ones. In some hypertrophic cells an early indication of transition to inactivity is suggested by the decreased number and length of microvilli whose disappearance may be related in this case to cell degeneration (Olson and Fromm, 1973). Moreover, some cells show more advanced signs of degeneration like reduced electron density and vacuolation of cytoplasm, while others are clearly degenerated with larger vacuolation and distal surfaces overlapped by projections of neighbouring cells.

However, such alterations do not occur at the same time in all gill filaments and this provides evidence of the maintainance of gill function (at least with regard to Cl^- uptake) in spite of acute nitrite intoxication. A similar phenomenon has been described for other toxicants: for example, in *Fundulus* exposed to Cd not all gill filaments are affected nor the pathological alterations induced by metal are localized in specific areas (Gardner and Yevich, 1970). Nevertheless, gill lamellae appear to be thinner in treated than in control trout especially because of the reduced diameter of blood vessels. This phenomenon may be due to the blood pressure drop induced by nitrite or to the contraction of cytoplasmic processes of pilaster cells involved in blood flow regulation (Morgan and Tovell, 1973). It has been assumed that this process—at the basis of the wide range of blood vessel size

Fig. 2. Light microscopic (a) and transmission electron (b)–(e) views of treated trout gills. (a) Semithin section showing some secondary lamellae characterized by a thinner central space and by protruding chloride cells. (b) Chloride cell bulging outward past the apical ends of the epithelial coast (arrow). (c) Apical cytoplasm of a bulging chloride cell with abundant granules (G) in the mitochondria free zone. M: mitochondria. (d) Chloride cell not overlapped by the ephithelial coat (EC) and showing an apical contact (arrow) with a cell protruding from an adjacent lamella. G: granules; N: nucleus; M: mitochondria. (e) Chloride cell supported by a peduncle (P) and completely projected outside. M: mitochondria; G: granules; N: nucleus.

Figure 3



(Newstead, 1967; Youson and Freeman, 1976)—may be influenced by variations in pO_2 and pCO_2 so representing a local response to toxicants in the aquatic environment (Newstead, 1967).

It is interesting to emphasize that chloride cells, in each stage of development, show intact mitochondria. Between all cell types, chloride cells are the richest in mitochondria (Philpott and Copeland, 1963) which represent the energetic source necessary for cellular ionic transport. Thus the integrity of mitochondria in spite of the acute nitrite intoxication may account for the unaffected ability in osmoregulation.

In this connection the liver mitochondria of trout treated with analogous nitrite concentrations constitute a target for the action of nitrite. The different susceptibility of gill and liver mitochondria support our hypothesis (Arillo *et al.*, 1984) that the marked nitrite hepatotoxicity is due to the peculiar hypoxic conditions of the liver of treated fish and/or the metabolic characteristics of this organ. The previous discussion indicates that chloride cells are involved in nitrite intoxication more than other gill cell types. This is in keeping with the hypothesis of Bath and Eddy (1980) on the function and importance of chloride cells in the high bioaccumulation of nitrite in freshwater fish.

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Fig. 3. Light microscopic (d) and transmission electron (a)–(c), (e) views of treated trout gills. (a) Cytoplasmic alterations in a chloride cell. CL: cytoplasmic lacunae; N: nucleus; M: mitochondria; EC: epithelial cells. (b) Degenerated chloride cell completely surrounded by epithelial cells (EC) and characterized by a wide cytoplasmic vacuolation. IB: inclusion bodies; M: mitochondria; WH: wide hole. (c) Lamella with reduced central space. (d) Semithin section of lamellae showing a slender shape. (e) Mitochondria (M) in the cytoplasm of a degenerating chloride cell.

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EFFECTS OF NUTRITION ON SEASONAL CHANGES IN TESTOSTERONE LEVELS IN YOUNG MALE REINDEER (*RANGIFER TARANDUS TARANDUS*)

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Abstract—1. Food restriction during autumn in male reindeer calves decreased peak testosterone levels at 4–6 months, and also at 16–17 months of age.

2. Peak testosterone levels at the age of 16–17 months were significantly correlated to body weight at 6 months but not to body weight at 16 months of age.

3. There were small secondary testosterone peaks (1–3 ng/ml) in March or April in 11 of 16 animals.

4. Testosterone levels started to increase before summer solstice in 50% of the yearlings.

INTRODUCTION

Whereas the effects of nutrition on fertility have been extensively studied in female mammals, such effects in males have received less attention. In more or less continuous breeders, restricted energy intake can reduce spermatogenesis or testosterone production (Salisbury and VanDemark, 1964; Leathem, 1975). On the other hand, overfeeding leading to excessive fat deposition may reduce fertility in bulls (Skinner, 1981).

Reindeer, like other northern ungulates, are seasonal breeders with a short mating period in autumn (Meschaks and Nordkvist, 1962). In summer, prior to the breeding season the animals grow rapidly and lay down large amounts of fat (McEwan, 1968; Dauphiné, 1976). This is especially prominent in the bulls. In the females, this fat deposition appears to be critical for reproductive performance (Thomas, 1982; Reimers, 1983). However, the effects of nutrition on reproduction in reindeer bulls have not been studied.

An experiment designed to study the effect of nutrition on growth hormone and thyroid hormones (Ryg and Jacobsen, 1982a) gave the opportunity to study also nutritional effects on testosterone levels in male reindeer calves, and also, within a limited range, the effect of differences in body weight of yearlings.

MATERIALS AND METHODS

The details of the experiment have been described earlier (Ryg and Jacobsen, 1982a). 16 male reindeer (*Rangifer tarandus tarandus*) calves were fed either reindeer lichen or a pelleted reindeer feed (RF 71) composed of 40% ground barley, 17% ground oats, 15% wheat bran, 3% soybean oil, and 25% ground grass, and containing 13.7% crude protein, 7.2% fat, 62.4% nitrogen-free extracts, 11% crude fiber, and 5.7% ash. From 23 August to 19 December, nine calves (here called group A) received RF 71 *ad libitum*, seven calves (group B) received restricted amounts (approximately maintenance ration) of RF 71. In group B, restricted feeding was continued until 12 February (two animals), 14 May (two animals) and 14 June (three animals). After these dates, RF 71 was allowed *ad libitum*. In group A, six animals were fed 60–70% of maintenance of RF 71 (three animals) or reindeer lichen (three animals) from 20 December to 12 February. Thereafter, RF 71 was admitted *ad libitum*. When correlating testosterone levels and body weights, I have included data for six animals from an earlier investigation (Ryg and Jacobsen, 1982b).

Blood samples were taken once or twice a month, by puncturing the *vena jugularis*. The blood serum was stored at −20°C until assayed. Serum testosterone was assayed using a radioimmunoassay as described by Sanwal *et al.* (1974) and modified by Sundby *et al.* (1975). The antiserum used crossreacts with 5_α-dihydrotestosterone (40%) and androstenedione (1.8%). The limit of detection was 0.2 ng/ml. Significance of differences between groups were tested using Student's *t*-test for unpaired data; $P < 0.05$ was regarded as significant.

RESULTS

In the calves, maximum testosterone levels were significantly higher in group A (2.2–5.5 ng/ml) than in group B (0.4–2.9 ng/ml). Peaks of 1.4–2.9 ng/ml occurred in four of the seven animals in group B, but later and less synchronous than in group A (Fig. 1). Also at 16–17 months of age, maximum testosterone levels during the autumn were significantly higher in group A (7.2–43.4 ng/ml) than in group B (9.7–11.0 ng/ml). Maximum body weights at this time were not significantly different in the two groups. There was a significant correlation between maximum testosterone levels in the yearlings and mean levels in the calves during the autumn ($r = 0.67$). When data from an earlier investigation (Ryg and Jacobsen, 1982b) are included, peak testosterone levels in the yearlings were weakly but significantly correlated ($r = 0.62$) to body weight at 6 months of age (Fig. 2a) but not to peak body weight at 16 months of age ($r = 0.42$, Fig. 2b). The partial correlation coefficient between peak testosterone and body weight at 6 months, with peak body weight at

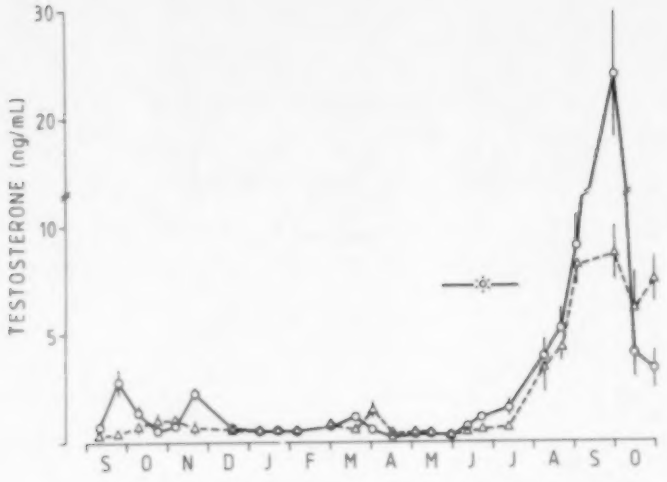

Fig. 1. Seasonal changes in serum testosterone levels in male reindeer calves with *ad libitum* (○) or restricted (△) feeding. Horizontal bars and "sun" indicate midnight sun and summer solstice. Notice change of scale on upper half or ordinate.

16 months held constant, was also significant ($r = 0.56$), whereas the partial correlation between peak testosterone and body weight at 16 months, with body weight at 6 months kept constant, was not significant (0.08).

Small testosterone peaks (1–3 ng/ml) also occurred in March or April in six of nine animals in group A, and in five of the seven animals in group B. These peaks tended to occur later in group B than in group A.

Although the most marked increases in testosterone levels in the yearlings only occurred after the end of the period of midnight sun (Fig. 1), there were persistent increases in many animals before summer solstice. Testosterone levels of 1 ng/ml or more were found earlier than 24 June in seven animals in group A, and in one animal in group B. This animal had been fed *ad libitum* since 12 February.

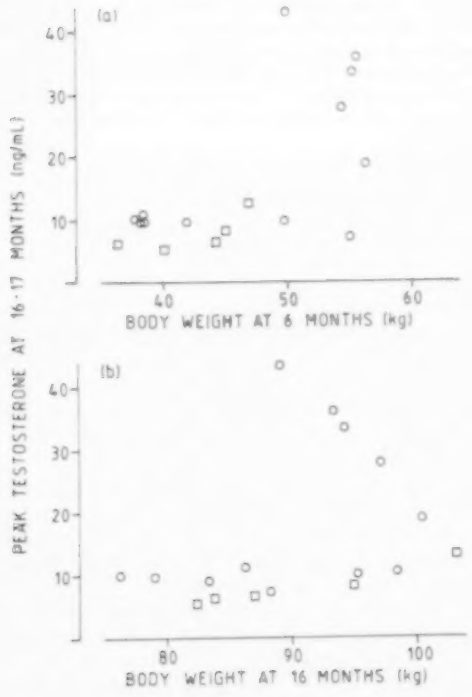

Fig. 2. Correlations between peak testosterone levels during rut in yearlings, and their body weight at the age of 6 months (a) and 16 months (b). □ Data from Ryg and Jacobsen (1982b). ○ This study.

DISCUSSION

The general pattern of testosterone levels found in this study, with androgenic cycles during autumn in both calves and yearlings, agrees with what has been reported earlier for reindeer and caribou (Whitehead and McEwan, 1973; Leader-Williams, 1979). The amplitude and timing of the testosterone peak in calves depended on feeding level; the peaks were lower and occurred later, if at all, in the restricted animals. This agrees with results from domestic ruminants (Leathem, 1975; Salisbury and VanDemark, 1964), and apparently also with results from red deer (Suttie, 1980).

Occasional activation of the hypophyseo-gonadal axis in early spring, as judged by the histology of the testes (Markwald *et al.*, 1971; West and Nordan, 1976), serum levels of gonadotropins (Bubenik *et al.*, 1982), testosterone (Whitehead and McEwan, 1973) or oestrogen (Bubenik *et al.*, 1979), or by urinary excretion of 17-ketosteroids (Meschaks and Nordkvist, 1962), seems to be a common occurrence in cervids. It is difficult to ascribe any function to such secondary reproductive peaks in reindeer. However, Goss (1976) found that a change in photoperiod—irrespective of the direction of the change—could initiate either growth or casting of antlers in sika deer, and Bubenik *et al.* (1982) suggested that secretion of gonadotropins might be initiated by a change

from decreasing to increasing daylengths, or vice versa, rather than by any particular daylength. The increases in testosterone levels before summer solstice, that occurred mostly in well-fed animals, could be due to endogenous factors. The existence of an endogenous, circannual rhythm involved in the antler cycle of deer was suggested by Goss (1969a,b); such a rhythm would presumably also affect the reproductive cycle.

The data presented here indicated that the testosterone levels of $1\frac{1}{2}$ year old males are not affected by their body weight before rut, but rather by their body weight as $\frac{1}{2}$-year olds (Figs 1 and 2). This result was surprising, and the interpretation should be regarded as tentative, because the comparison between groups A (calves fed *ad libitum*) and B (restricted) was not planned, and because there was a considerable overlap between peak testosterone levels. Also, Suttie (1980) found no effect of restricted feeding during winter on testosterone levels of yearling red deer. However, the point warrants further study, because if the interpretation presented here is correct, it has implications for reindeer husbandry. Testosterone influences social and sexual behaviour of deer; animals with high testosterone levels tend to be dominant (Lincoln *et al.*, 1972; Stokkan *et al.*, 1980). Supplementary feeding, or other ways of increasing the body size of male calves in the autumn, could increase the value of yearlings as breeding animals.

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EFFECT OF TIME AND DIET ON CARCASS FATTY ACID COMPOSITION OF BOBWHITES, *COLINUS VIRGINIANUS*

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Abstract—1. Fatty acid composition of starved bobwhites returned to prestarved profiles within 20–30 days as bobwhites on a recovery diet regained lost weight.

2. Percent composition of at least 50% of carcass fatty acids was significantly different from that of fatty acids in the bobwhite's diet.

3. Nutritionally deprived bobwhites had a higher percent composition of arachidonic acid (20:4) than did birds on a nutritious diet.

4. Bobwhites altered the fatty acids of most diets in a consistent manner.

5. Discriminant analysis of carcass fatty acid composition correctly categorized 81% of the birds into their actual 20-day dietary treatment.

INTRODUCTION

Avian depot fats exhibit fatty acid profiles characteristic of season, diet, species, sex, and individual. Studies of the influence of diet on avian fatty acid profiles have produced conflicting results. Seasonal differences in the fatty acid composition of wild birds have been ascribed, at least in part, to seasonal differences in diet (Moss and Lough, 1968; West and Meng, 1968b; Barnett, 1970; Ijas *et al.*, 1978). Yom-Tov and Tietz (1978) concluded that diet affected the fatty acid composition of depot fat of rock partridge, *Alectoris chucar*, feeding on either sorghum seeds or olives in the same season. In contrast, dietary fatty acids were not found to influence the fat composition of the red poll, *Acanthis flammea* (West and Meng, 1968a), crossbills, *Loxia curvirostra* and *L. leucoptera* (Tanhuanpaa and Pullianen, 1975), and domestic fowl (Hazelwood, 1972).

Most of the preceding studies involved direct statistical comparisons of the fatty acid content of the birds to that of their diets. However, the absence of statistical equality between the two fatty acid profiles does not mean that diet does not influence the fatty acid profile of an organism. Even if birds alter dietary fats, there may be a relationship between bird and diet fatty acids.

Discriminant analysis may be a more appropriate statistical indicator of dietary influences on depot fatty acid composition than is analysis of variance. Discriminant analysis differentiates among groups and places individuals into specific categories (e.g. dietary) by simultaneously analyzing two or more variables (Snedecor and Cochran, 1967; Sokal and Rohlf, 1969).

The purpose of this paper is to report the effect of time and diet on the fatty acid compositions of bobwhites, *Colinus virginianus*, under controlled laboratory conditions using both analysis of variance and discriminant analysis.

MATERIALS AND METHODS

Weight stabilization

Bobwhites used in this study were adult males obtained during winter from the Kansas Fish and Game Commission Quail Farm at Pittsburg, Kansas. Bobwhites were individually confined in 48 × 25 × 13 cm polypropylene cages with 1.3 cm hardware cloth tops, and were housed in an environmental chamber with 9L:15D photoperiod, 5°C temperature, and 85% relative humidity. Birds were provided a balanced mash (P-18) and water *ad libitum* for at least 30 days until their individual weights varied by less than ± 2% of their individual mean weights during the previous 15 days.

Experimental treatments

For each of two experiments, 30 weight-stable birds were randomly divided into six 5-bird weight stratified groups. At the beginning of each experiment, one 5-bird group was selected at random and killed for fatty acid analysis. The other 5-bird groups were then starved to 75% of their initial stable weight. The first experiment studied the effects of time on fatty acid composition. One of the starved groups was killed and the remaining 4 groups were refed the P-18 mash *ad libitum* and killed at the end of 10, 20, 30, or 40 days. The second experiment studied the effect of diet on fatty acid composition. One starved group was killed and the remaining four groups were provided seeds of milo, *Sorghum vulgare*, sunflower, *Helianthus maximiliani*, soybean, *Soja max*, false sunflower, *Heliopsis helianthoides*, or sumac, *Rhus glabra*, *ad libitum* for 20 days then killed for fatty acid analysis. Carcasses of all killed birds were stored frozen until analyzed for fatty acid composition.

Fatty acid analysis

Head, wings, feet, skin and crop contents were removed from each frozen carcass, leaving subcutaneous fat deposits on the carcass. Carcasses were weighed to ± 0.1 g and processed through a food chopper (Beardmore and Robel, 1976). Individual processed carcasses were blended in 300 ml of chloroform–methanol (2:1 v/v), vacuum filtered, and the debris reblended with another 200 ml of chloroform–methanol (modified from Folch *et al.*, 1957). Each combined chloroform–methanol solution was washed

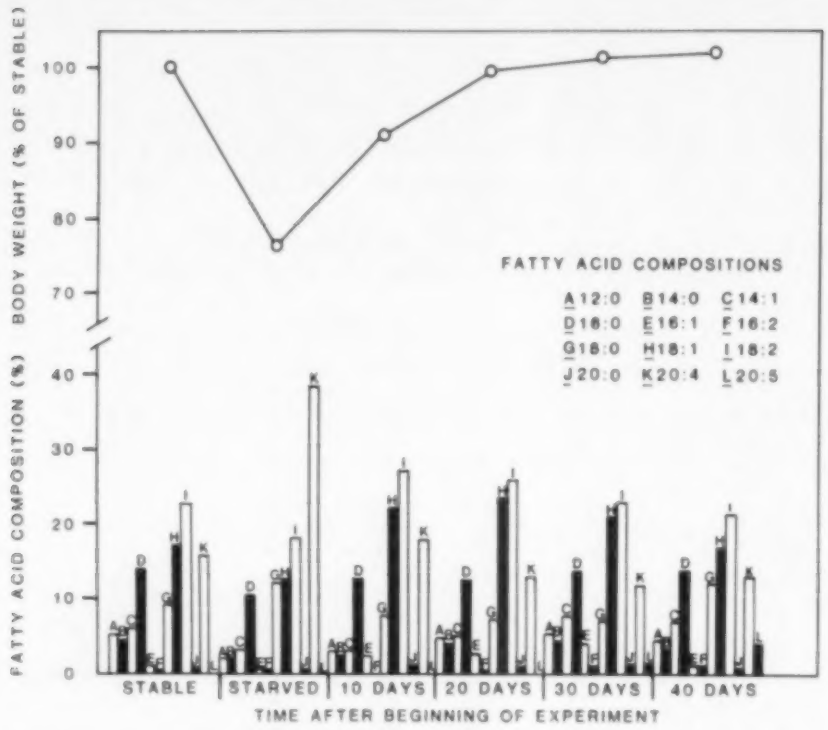

Fig. 1. Percentage composition of carcass fatty acids and body weights of bobwhites during prestarved (stable), starved, and 10-day recovery phases of this experiment.

twice, each time with 250 ml of deionized distilled water. Dietary lipids were extracted from ground seed using the method, with no modifications, from Folch *et al.* (1957).

Methyl esters were formed from the concentrated extracted lipids by adding 5 ml of boron trichloride in methanol, shaking the mixture and allowing it to set for 1 hr at room temperature, then adding hexane. Methyl esters collected from the upper layer were analyzed by gas–liquid chromatography on columns of diethylene glycol succinate at 170–180 C. The percent fatty acid composition of the mixture was calculated using peak-height times the width at one-half height (Heftman, 1967). Fatty acids not identified by comparison with standards were identified on a plot of the log retention times of known fatty acids against their carbon numbers and compared to Table II of Hofstetter and Holman (1965: 539).

Statistical analysis

Analysis of variance (ANOVA) and least squares separation of means were used for direct comparison of bird and dietary fatty acids. Not all fatty acid data could be used in the ANOVA due to differences in their variances. Discriminant analysis was used for assigning bobwhites into diet categories based on carcass fatty acids only. The statistical computer package SAS (Helwig and Council, 1979) was used. Statistical significance was $P = 0.05$.

RESULTS AND DISCUSSION

Effect of time

Bobwhites starved to 75% of their pretrial body weights regained their lost weight after 20 days on an *ad libitum* recovery diet of P-18 (Fig. 1). Starved bobwhites had lower percentages of 12:0, 14:0, 16:0, 16:1, 18:1, and 18:2 fatty acids than the prestarved group. The relative amounts of five of these fatty acids returned to the prestarved normal within 20–30 days and 16:1 returned by 40 days. Their fatty acid composition at 40 days was not significantly different from that of the weight-stable bobwhites (Fig. 1). The percentages of fatty acids 18:0 and 20:4 increased in starved bobwhites, but also returned to normal after 10–20 days as the birds regained their prestarvation weight. A dramatic percentage increase of arachidonic acid 20:4 might be diagnostic of starved bobwhites. The consistency of the new fatty acid profile with the original profile suggests a causal relationship between dietary fats and their alteration into body fats.

Effect of diet

Fatty acid profiles of bobwhites recovering their weight were significantly different in 2–5 of the six diet/carcass fatty acid comparisons, but there was no consistent pattern in the fatty acids that differed (Table 1). Fatty acid composition also differed between bobwhites provided different recovery diets for 20 days (Table 2). Most of these differences occurred in the level of fatty acids 18:1, 18:2 and 20:4. These fatty acids constitute from 55.7 to 70.9% of the overall fatty acid profile.

Carcasses of starved bobwhites and those birds provided a diet of sumac or false sunflower had very high levels of arachidonic acid (20:4). Bobwhites on false sunflower or sumac were not able to regain weight and survived no longer than 6 days into the

Table 1. Mean percent fatty acid compositions of dietary items and of carcasses of bobwhites that were on those diets during a 20-day weight recovery period

Dietary treatment	N	Percent fatty acid (carbon:unsaturated) 16:0	18:0	18:1	18:2*	Saturated*	Unsaturated*
P-18	2	16.5†	2.4†	31.7†	54.3†	18.6†	81.4†
Carcass	5	15.7†	7.5‡	17.2‡	22.6‡	40.0‡	60.0‡
Milo	2	16.7†	2.7†	39.0†	51.2†	17.8†	82.7†
Carcass	5	11.4†	12.3‡	18.9‡	23.4‡	29.0‡	68.6‡
Sunflower	2	10.6†	4.4†	13.0†	73.9†	14.2†	85.8†
Carcass	4	10.9†	15.0‡	9.3†	45.5‡	29.3‡	71.3‡
Soybean	2	24.1†	14.4†	28.1†	44.5†	34.7†	68.2†
Carcass	3	14.9‡	12.1†	19.1†	25.1‡	32.0†	68.0†
False sunflower	2	10.0†	5.5†	35.0†	56.8†	14.8†	85.2†
Carcass	7	14.7†	11.6†	12.9‡	18.5‡	26.3‡	73.6‡
Sumac	2	23.5†	16.9†	36.0†	25.6†	44.6†	55.4†
Carcass	6	12.1‡	11.8†	10.3‡	11.8‡	26.8‡	73.2‡

*Statistical analysis performed on raw data. All other data arcsine transformed.
†‡ = Least square mean values with common superscripts within a column in a section are not significantly different ($P = 0.05$).

20-day weight recovery period. The high percentage of arachidonic acid in these food-stressed birds may have reflected preferential retention of this essential fatty acid while other fatty acids were being utilized. This preferential retention supports observations of Suttie (1972: 57) and Hilditch (1956: 478) who pointed out the necessity of arachidonic acid for proper growth and cell maintenance. Arachidonic acid is not a major component of the triglycerides, which are the major lipids used for energy reserve (Gunstone, 1958: 19).

Unsaturated fatty acids constituted the majority of the fatty acids of bobwhites throughout this study (Table 1). While Bower and Helms (1968) showed the absolute energetic gain of selectively storing unsaturated fats to be of minor adaptive significance, Johnson and West (1973) proposed that the increased mobility of unsaturated fats might be of adaptive significance during the metabolic demands of migration. The increased mobility of unsaturated fatty acids may also be important for non-migratory birds during periods of food deprivation and/or extreme environmental conditions, when energy reserves need to be mobilized quickly to maintain homeostasis in ambient temperatures below the thermoneutral zone (Geers and Decuypere, 1978).

Perhaps all that may be concluded from these comparisons is the degree to which the bird's fatty acid composition resembles that of the diet. The question of whether or not diet influences the fatty acid composition of birds is not answerable by such analyses. It is known that dietary lipids are broken down in the small intestine and also are synthesized from nonlipid compounds (Hazelwood, 1972).

Identification of diet using discriminant analysis

Discriminant analysis of carcass fatty acid composition correctly categorized all (23) of the bobwhites provided diets of P-18, milo, false sunflower, or sumac. The analysis was also able to correctly categorize two of four birds on the sunflower diet, and one of three birds on the soybean diet (Table 3). Thus, use of discriminant analysis on carcass fatty acid compositions allowed the correct classification of 26 of 32 (81.25%) bobwhites into their dietary groups. This successful determination of specific diets

Table 2. Mean percent fatty acid compositions of prestarved (stable) and starved bobwhites, and of bobwhites at the end of 20 days on specific weight recovery diets

Fatty acid (carbon:unsaturated)	Experimental group: Weight stable	Starved	Recovery diet: Milo	Sunflower	Soybean	False sunflower	Sumac
12:0	10.0†	1.6‡	2.1‡	1.6‡	2.4‡	2.3‡	2.1‡
14:0	9.4†	1.4‡	2.3‡	2.2‡	1.9‡	1.4‡	1.4‡
14:1	5.5†	1.4‡	1.4‡	2.4‡	2.4†‡	2.9†‡	2.4‡
16:0	15.7†	15.2†	11.4†	10.9†	14.9†	14.7†	12.1†
16:1	0.1†‡	0.1†‡	5.6‡	0.1†‡	0.0†	0.0†	0.0†
16:2	0.0†	0.0†	0.0†	0.5†	0.1†	0.1†	0.0†
18:0	7.5†	14.5†‡	12.3†‡	15.0‡	12.1†‡	11.6†‡	11.8†‡
18:1	17.2†‡	14.1†‡§	18.9†	9.3§	19.1†‡	12.9‡§	10.3§
18:2*	22.6†‡	14.7‡§	23.4†‡	45.5‖	25.1†	18.5†‡	11.8§
20:0	0.0†	0.0†	0.2†	0.1†	0.4†	0.0†	0.0†
20:4*	15.9†‡	39.6§‖	25.6†	13.8‡	21.7†‡	39.3§	48.8‖
N	5	2	5	4	3	7	6

*Statistical analysis performed on raw data. All other data were arcsine transformed.
†‡§‖ = Least square mean values with common superscripts within a row are not significantly different ($P = 0.05$).

Table 3. Discriminant analysis assignment of bobwhites into dietary categories using carcass fatty acid composition of starved birds on specific recovery diets for 20 days

Dietary treatment	Number of birds	Categorized into	Probability of correct categorization
P-18	5	P-18	0.9999
Milo	5	Milo	0.9534
False sunflower	7	False sunflower	1.0000
Sumac	6	Sumac	0.9999
Sunflower	2	Sunflower	1.0000
	2	Sumac	0.8984
Soybean	1	Soybean	0.9984
	2	Milo	0.9928
Starved	1	Milo	0.9950
	1	Sumac	0.9330

from fatty acid compositions of carcasses reflects a relationship between the fatty acid profile of the diet and the carcass.

As noted in Table 1, the fatty acids of the diet are not simply absorbed and stored unaltered in the bobwhite carcass. Variations did exist between bobwhites in different treatments, and the only factor that differed in these treatments was their diet. Therefore, diet must have influenced the fatty acid profile of the bobwhites. Discriminant analysis results indicate that bobwhites produce a specific fatty acid profile when metabolizing certain dietary items. In this study, fatty acid profiles were unique for diets of P-18, milo, false sunflower, and sumac, but not unique for diets of sunflower and soybean, or for the starved birds. Half of the latter were misclassified as sumac. Both starved birds and sumac-fed birds were malnourished, based on weight loss and survival.

Additional use of discriminant analysis in the study of fatty acid compositions may provide insights into the biological significance of different diets in the survival of food-stressed wild birds.

Acknowledgements—We are grateful to A. D. Dayton for statistical consultation, and W. Klopfenstein for use of the gas–liquid chromatograph and interpretation of data. The study was financed by the Kansas Agricultural Experiment Station and the National Science Foundation (Grant No. BSR-8022568). Contribution No. 83-252-J, Division of Biology, Kansas Agricultural Experiment Station, Kansas State University, Manhattan, KS 66506.

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THE EFFECTS OF HYPOXIA ON BLOOD pH AND LACTATE LEVELS IN *MACROBRACHIUM ROSENBERGII* (DE MAN)

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Abstract—1. *Macrobrachium* blood pH increases from 7.40 to 7.81 between 80 and 40 torr due to a hyperventilatory response. This change in pH helps to sustain a substantial level of oxygen transport at 40 torr via the Bohr effect.

2. At 15 torr, blood pH decreases to 7.34 and lactate accumulates.

3. At normoxia, blood lactate measures 0.31 ± 0.08 mM/l blood, and after 2 and 6 hr exposure to 40 torr increases to 0.98 ± 0.05 and 0.60 ± 0.05 mM/l blood, respectively.

4. When animals are placed at 15 torr for 2 hr, blood lactate levels increase to 12 ± 0.55 mM/l blood, and upon return to normoxic conditions, blood lactate levels are comparable to normoxic controls within 3 hr.

INTRODUCTION

Hypoxia with its consequent effects on oxygen transport may effect the respiratory physiology of crustaceans in one of two ways. Species may either oppose the impingement on their oxygen transport by modifying their circulatory and/or ventilatory systems (McMahon *et al.*, 1974; Hill and Koopowitz, 1975; Taylor, 1976; McMahon *et al.*, 1978; Butler *et al.*, 1978; Batterton and Cameron, 1978; de Fur and Mangum, 1979; Burnett, 1979), or they may suffer an oxygen deficiency to the tissues with a lactate accumulation.

The ability to modify their circulatory and/or ventilatory systems is, however, limited. When the degree of hypoxia goes beyond the tolerance limit for a given species, there is a subsequent suppression of oxygen uptake and lactate accumulation. With these changes in ventilation and circulation, corresponding changes also occur in blood pH and carbon dioxide tensions (Burnett, 1979; Cameron and Mangum, 1983).

In the present study, the effects of hypoxia on blood pH, ventilation and lactate accumulation were investigated in the Pacific freshwater prawn. *Macrobrachium rosenbergii*.

MATERIALS AND METHODS

Animals were obtained from the Anuenue Fisheries Research Center, Sand Island, Honolulu, Hawaii and were maintained in the laboratory at 22°C. Animals weighed between 50 and 75 g and were starved for 48 hr prior to all experimentation.

For the purpose of experimentation, animals were placed into 4 l. tanks for indicated time periods in which the oxygen tension of the water was closely regulated. Water oxygen tension was monitored with a Yellow Springs Instrument Co. polarographic electrode (5420A) and the required oxygen tension obtained by bubbling either air or nitrogen into the water.

Since the scaphognathite may be observed through the carapace, measurement of beating frequencies were directly made during experiments on ventilation. Although quantitative measurements of water flow over the gills were not taken, scaphognathite beating frequencies are a good indicator of ventilatory activity.

Blood pH was determined with an Orion implantable pH electrode (916300). The electrode was directly inserted into the ventral muscle of the first abdominal segment and the pH values read with a Leeds and Northrup pH meter (7405).

Blood lactate levels were determined in 0.5 ml of prebrachial blood obtained via a hypodermic syringe through the base of the fourth or fifth walking leg. In some cases, blood samples were pooled due to the difficulty in obtaining enough blood from individuals. Blood samples were analyzed with a Sigma lactate test kit, modified according to the procedures given in Graham *et al.* (1983).

RESULTS

Effects of hypoxia on blood pH and ventilation

Below 80 torr there is a rapid change in blood pH with hypoxia (Fig. 1). Blood pH increases from 7.40 to 7.81 between 80 and 40 torr and decreases between 40 and 15 torr to 7.34. Over the same oxygen tension range scaphognathite beating frequency increases (Fig. 2). This hyperventilatory response increases blood pH by the loss of carbon dioxide, i.e. lowering of blood P_{CO_2}. Similar responses have been observed for other decapod species (Burnett, 1979; Sinha and Dejours, 1980). These changes in blood pH occur rapidly and suggest a rapid adjustment to hypoxia conditions (Fig. 3). Since elevated pH increases hemocyanin oxygen affinity, the change in blood pH allows the blood to maintain its oxygen transport function at a level comparable to normoxia (Mauro and Malecha, 1984). *Macrobrachium* is, however, limited in its ability to compensate to hypoxia as indicated by the drop in both pH and oxygen uptake below 40 torr (Mauro and Malecha, 1984).

Lactate production with hypoxia

At normoxia blood lactate levels are low (0.31 ± 0.08 mM/l blood) and range from 0 to

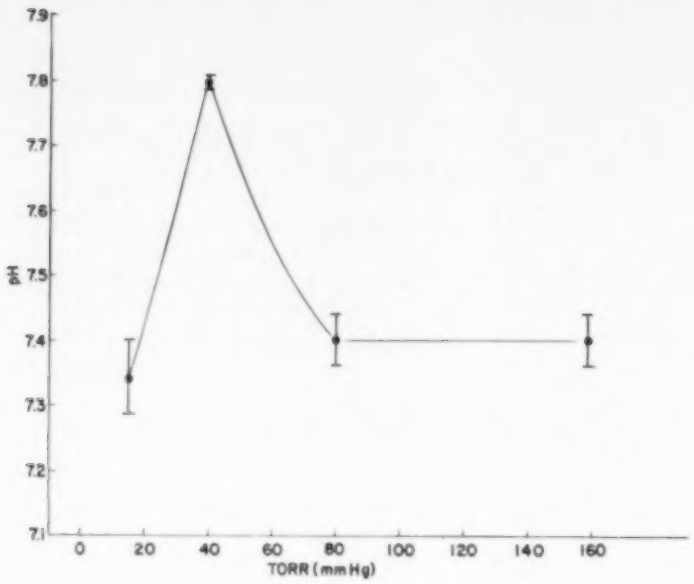

Fig. 1. Blood pH after a 2 hr exposure to different oxygen tensions. Mean ± SEM = 6–12 for each oxygen tension.

0.56 mM/l blood (Fig. 4). After 2 and 6 hr exposure to 40 torr the increase in blood lactate was small and measured 0.98 ± 0.05 mM and 0.60 ± 0.05 mM/l blood, respectively. Since adequate oxygen transport is maintained to 40 torr, delivery of oxygen to the tissues is sufficient to fuel aerobic cellular respiration and impede lactate production. At 15 torr, oxygen transport decreases the lactate accumulates (Fig. 4). After 2 hr at 15 torr, blood lactate accumulated to 12 ± 0.55 mM/l blood. This high level of lactate was

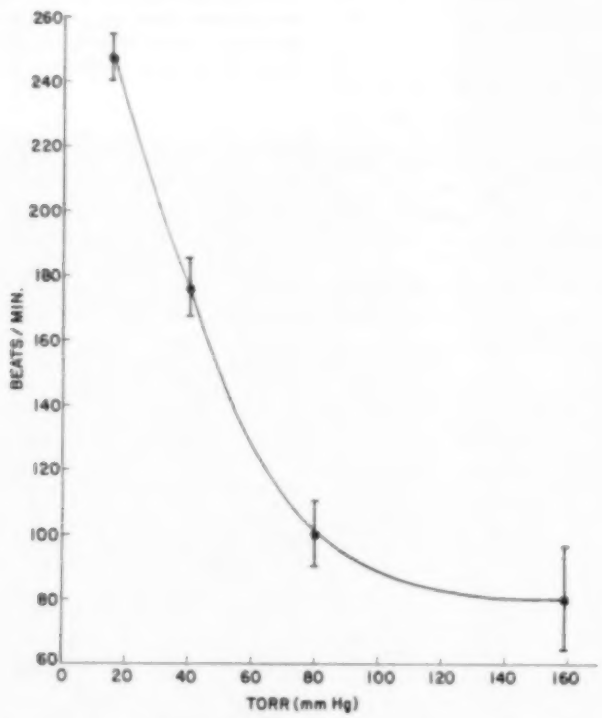

Fig. 2. Change in scaphognathite beating frequency with oxygen tension. Mean ± SEM = 8–12 for each oxygen tension.

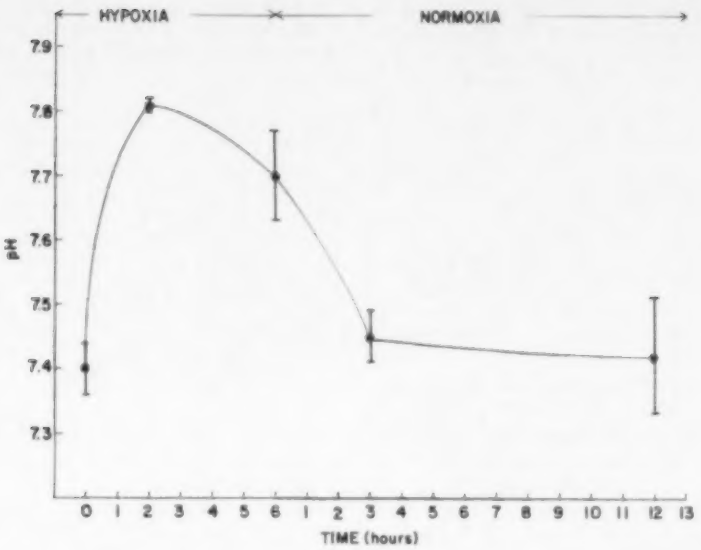

Fig. 3. Blood pH during 6 hr exposure to 40 torr followed by recovery at 159 torr. Mean ± SEM = 6–12 for each exposure.

maintained after 6 hr and measured 9.4 ± 0.30 mM/l blood. Upon return to normoxia, blood lactate levels fall rapidly. After 3 hr re-exposure to normoxia, blood lactate measured 0.54 ± 0.18 mM/l blood. This value is comparable to that observed for normoxia controls. At 9 hr re-exposure to normoxia, blood lactate levels were measured at 0.17 ± 0.09 mM/l blood.

DISCUSSION

Hypoxia induction of hypocapnic alkalosis in *Macrobrachium rosenbergii* is comparable to that reported for other species of crustaceans (Burnett, 1979; Sinha and Dejours, 1980). The change in pH is small (Δ0.41), but is significant in relation to its effect on hemocyanin oxygen affinity. With such an increase in pH, P_{50} would decrease from 28 torr to below 5 torr (Mauro and Malecha, 1984). This increase in oxygen affinity permits a high oxygen uptake by prebranchial blood at the gill and thus an adequate supply of oxygen to the tissues despite a reduced oxygen tension gradient. Between 40 and 159 torr there is no appreciable lactate accumulation and oxygen uptake is maintained. Below 40 torr, blood lactate accumulates and aerobic respiration is de-

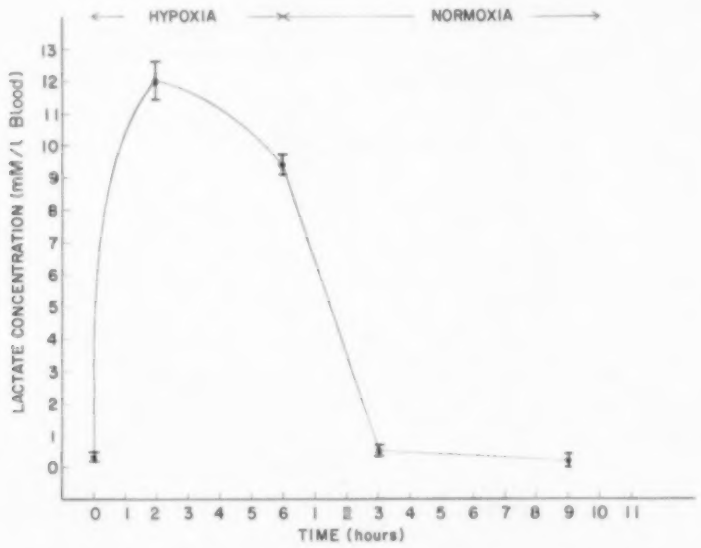

Fig. 4. Lactate production during 6 hr exposure to 15 torr followed by recovery at 159 torr. Mean ± SEM, $N = 6$, for each exposure.

pressed. When returned to normoxia after 6 hr exposure to hypoxia, reduction in blood lactate is rapid. The speed of this response is in marked contrast to *Galathea stigosa* or *Homarus gammarus* in which the removal of lactate from the blood is relatively slow (Bridges and Brand, 1980). Whether the differences in the rate of removal of lactate from the blood are due to differences in metabolic rates or to differences in the relative activities of their glyconeogenic pathways was not ascertained in this study.

The data obtained on *Macrobrachium* suggested that this species is well adapted to hypoxia by its maintenance of oxygen uptake at low oxygen tensions and also by the rapidity of its lactate removal from the blood.

Spotts (1983) has questioned the use of hemolymph lactate concentrations for determining the effectiveness of anaerobic compensation during hypoxia. This position is based on the slow rate which he infers lactate enters the blood and an underestimated importance of hemolymph lactate to oxygen transport. Quoting Phillips *et al.* (1977) he states that lactate can take as long as 6 hr to pass into the hemolymph. He, however, does not mention that Phillips *et al.* (1977) also observed the maximum increase in blood lactate titer after 50 and 25 min completion of exercise in the yabbie and lobster, respectively. In regard to oxygen transport, hemolymph lactate is important to oxygen transport as both a contributor to blood pH and as a possible modulator of oxygen affinity (Graham *et al.*, 1983). Since the tissues can use only the oxygen which is delivered to them by the blood, factors which modify the oxygen transport characteristics of the blood are very important in determining the effectiveness of anaerobic compensation during hypoxia.

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INTRASPECIFIC COMPARISON OF HEMOCYANIN OXYGEN AFFINITY AND THE EFFECTS OF HYPOXIA ON OXYGEN CONSUMPTION IN *MACROBRACHIUM ROSENBERGII* (DE MAN)

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Abstract—1. No significant difference ($P > 0.5$) was observed in the oxygen binding characteristics of three strains of *M. rosenbergii* hemocyanin. P_{50} was equal to 19.6 ± 0.5 at a pH of 7.54, $n_{50} = 5.2 \pm 0.2$ at 22°C.

2. The Bohr effect ($\Delta \log P_{50}/\Delta pH$) for *M. rosenbergii* hemocyanin is -1.3.

3. Cooperativity (n_{50}) decreased with increasing pH from 7.3 ± 0.9 at pH 7.36 to 1.2 ± 0.1 at pH 7.71.

4. Among different strains, no difference ($P > 0.5$) was observed on the effects of hypoxia on oxygen uptake.

5. Although large variations in morphology, protein structure, reproduction, and behavior are observed among the different strains of *M. rosenbergii*, the characteristics of the respiratory system appear to be extremely conservative.

INTRODUCTION

The freshwater prawn *Macrobrachium rosenbergii* (de Man) is widely distributed across the South Pacific. Within its range the species is neither morphologically nor genetically homogenous (Malecha, 1980; Malecha *et al.*, 1982). On these criteria, *Macrobrachium* has been divided into two major groups: the Western Pacific and the Eastern Pacific. The latter is divided into the North Eastern Pacific and the Australian groups. In addition, each group is unique in its behavioral and reproductive characteristics. These variations are so large as to suggest the presence of a group of closely related species rather than a single species comprising many races.

While the electrophoretic, morphological, and behavioral differences with *Macrobrachium* are well documented (Malecha, 1980, Malecha *et al.*, 1982), neither the intraspecific effects of hypoxia on oxygen consumption nor the hemocyanin oxygen affinity have been studied. These parameters would help assess whether the observed intraspecific variation in *M. rosenbergii* is comparable for its respiratory physiology. The present study examines these parameters in three strains of *M. rosenbergii* representative of its major geographic groupings.

MATERIALS AND METHODS

Animals from the three major geographic groupings were obtained. To represent an Eastern Pacific, an Australian, and a Western Pacific group, a Philippine strain, an Australian strain, and an Anuenue strain (Malaysian) were used respectively. These pure strains were maintained and raised at the Anuenue Fisheries Research Center, Sand Island, Honolulu, HI.

Prior to experiments on oxygen uptake, animals were maintained in the laboratory for a period of two weeks at 22°C and starved for 48 hr before being tested. Oxygen uptake ($\dot{V}_{O_2}$) of animals in the dark were determined by the depletion of oxygen in closed containers (Yellow Springs Instrument Co. 5420). These animals were allowed to adjust to aerated containers for 30 min before the beginning of measurements and their weights ranged from 4 to 15 g.

Oxygen equilibrium properties of diluted blood were determined tonometrically. Whole blood samples were allowed to clot and the serum collected after centrifugation. The serum was diluted with Bis–Tris (0.05 M) buffered *Macrobrachium* Ringers (Ahern *et al.*, 1977), pH adjusted with HCl at 22°C. Premixed and analyzed O_2–N_2 mixtures were purchased from Arco Company, Santa Clara, California. These mixtures were equilibrated with diluted blood samples and the change in absorbance read at 345 nm (Bausch and Lomb Spectronic 20 Colorimeter). The gases were humidified before exposure to the diluted blood samples in 1 l bottles and the equilibration period for exposure chosen by ascertaining no further change in absorbance after 5 min. This period was approx 10–15 min at each P_{O_2}.

Data presented as mean values (±standard error) and were compared according to Student's *t*-test using unpaired observations.

RESULTS

Hemocyanin oxygen equilibrium properties

Among the different strains, no significant difference ($P > 0.5$) was observed for the hemocyanin oxygen binding characteristics. When compared at 22°C and a pH of 7.54 ($N = 6$ for each strain), P_{50} was equal to 19.9 ± 0.8, 20.0 ± 0.9 and 19.0 ± 0.8 for the Anuenue, Philippine, and Australian strains, respectively.

At 22°C, the oxygen affinity of *M. rosenbergii* hemocyanin is strongly pH dependent with the Bohr effect ($\Delta \log P_{50}/\Delta pH$) equal to -1.3 in the pH range 7.36–7.71. P_{50} measures 30.4 ± 0.7 ($N = 6$), 19.6 ± 0.5 ($N = 18$), and $9.0 \pm 0.6 \pm 0.6$ ($N = 6$) torr (mmHg) at pH 7.36, 7.54, and 7.71, respectively. Over the same

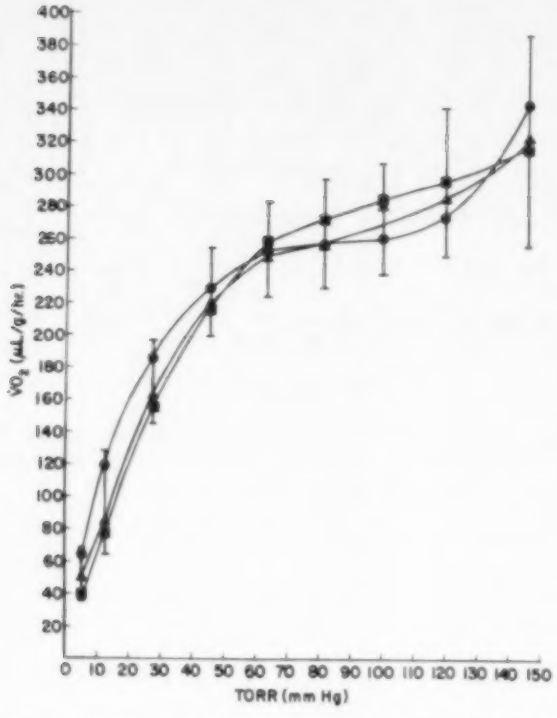

Fig. 1. Effects of hypoxia on oxygen uptake in three strains of *M. rosenbergii* at 22°C. Data expressed as means ($N = 6$) with the maximum range in error for the three strains represented, $\dot{V}_{O_2}$ in μl/g wet wt/hr. Anuenue (▲), Australian (■), Philippine (●).

pH range, cooperativity (n_{50}) decreases with the increase in pH. It measures 7.3 ± 0.9 at pH 7.36, 5.2 ± 0.2 at pH 7.54 and 1.2 ± 0.1 at pH 7.71. These properties are not unusual for crustacean hemocyanins and are very typical for decapods (Mangum, 1983).

Effect of hypoxia on oxygen uptake for Anuenue, Australian and Philippine stocks

Among the various stocks of *M. rosenbergii* no significant differences ($P > 0.5$) were observed in oxygen uptake when exposed to hypoxia (Fig. 1, Table 1). This species regulates its oxygen uptake to about 60 torr ($P_{c O_2}$) where it decreases to a low value of 40–60 μl/g wet wt/hr at 5 torr. These results suggest that ventilatory and circulatory adaptations to hypoxia are comparable among the different strains.

Table 1. Effects of oxygen tension (P_{O_2}) on oxygen uptake ($\dot{V}_{O_2}$) by three strains of *Macrobrachium rosenbergii*.

P_{O_2}	Strain Anuenue	Phillippine	Australian
145	322 ± 32	344 ± 43	315 ± 59
119	285 ± 28	274 ± 24	297 ± 45
99	280 ± 21	260 ± 22	284 ± 23
81	257 ± 27	257 ± 25	272 ± 25
63	249 ± 25	253 ± 24	258 ± 25
45	220 ± 20	229 ± 25	217 ± 14
27	163 ± 17	185 ± 12	157 ± 12
13	84 ± 8	119 ± 9	78 ± 14
5	50 ± 5	64 ± 4	39 ± 1

Mean ± SE of six observations on each of six intact animals. $\dot{V}_{O_2}$ expressed in μl/g wet wt/hr.

DISCUSSION

Among the strains of *M. rosenbergii*, respiratory physiology does not appear to vary. No variation is seen in the oxygen binding properties of hemocyanin between strains with a similar absence of variation for the effects of hypoxia on oxygen uptake. This lack in intraspecific variation was not unexpected. As a group, crustaceans are extremely conservative with respect to the structure and function of their respiratory proteins as well as the characteristics of their respiratory systems (Mangum, 1983).

Most crustaceans have hemocyanin as their respiratory protein. This molecule does not appear to vary greatly in different species qualitatively or quantitatively. According to Mangum (1983) this may account for the more conservative functional properties of this molecule in comparison with other oxygen carriers.

Since hemocyanin oxygen carrying properties do not vary extensively among crustaceans as a group, the lack of intraspecific variability for *M. rosenbergii* hemocyanin is predictable. Less predictable, however, is the consistent effect of hypoxia on oxygen uptake. In its natural habitat, the Australian strain of *M. rosenbergii* is exposed to low oxygen tensions for prolonged periods more often than the other strains. One might expect to see greater tolerance to hypoxia in this strain. For example, in the fish *Cyprinodon nevadensis* increased hypoxia tolerence is associated with the subspecies more frequently exposed to low oxygen tensions (Hirshfield *et al.*, 1980). However, in *Macrobrachium* no differences were observed in hyp-

oxia effects on oxygen uptake among the different strains.

Either the comparatively greater exposure in the Australian strain has not occurred for an evolutionary time span long enough to permit selection of improved hypoxia adaptations, or this species is presently at its best adapted state and is limited in its capacity to improve by structural and/or molecular limitations.

An alternative hypothesis supported by these data is that not all portions of the crustacean genome are equally responsive to evolutionary change, i.e. that those elements and mechanisms associated with respiration are highly conservative and lacking in evolutionary plasticity.

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β 1,3-GLUCAN INDUCED CELLULAR DEFENCE REACTIONS IN THE SHORE CRAB, *CARCINUS MAENAS*

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Abstract—1. A marked reduction in the number of circulating haemocytes concomitant with the formation of discrete haemocyte clumps in the gills was induced in *C. maenas* by injection of β 1,3-glucans, but not other soluble glycans.

2. The haemocytes were very sensitive to the presence of the glucans, with doses as low as 10^{-7}g/crab still effective in diminishing the cell count.

3. Since β 1,3-glucans are known to specifically activate the prophenoloxidase activating system within the haemocytes, these findings offer further support for the role of this pathway in non-self recognition by crustaceans.

INTRODUCTION

β 1,3-Glucans, soluble carbohydrates from the cell walls of fungi and algae, are known to activate the alternative pathway of complement (Hamuro *et al.*, 1978; Reid and Porter, 1981) and to have a potent stimulatory effect on the reticuloendothelial system of mammals (Kokoshis *et al.*, 1978; DiLuzio, 1979; Seljelid *et al.*, 1981). They also induce gelation of *Limulus* amoebocyte lysate (Kakinuma *et al.*, 1981; Morita *et al.*, 1981) and, in crustaceans, trigger melanization of the haemolymph by activating prophenoloxidase within the haemocytes (Unestam and Söderhäll, 1977; Söderhäll and Unestam, 1979; Söderhäll, 1981). This activation of prophenoloxidase by the glucans is highly specific, involves a complex enzyme cascade (Söderhäll, 1982) and converts prophenoloxidase to the "sticky" protein, phenoloxidase (Söderhäll *et al.*, 1979; Söderhäll, 1981). With viable haemocytes from the crayfish, *Astacus astacus*, and the shore crab, *Carcinus maenas*, we have recently found that soluble β 1,3-glucans significantly enhance the rate of phagocytosis of the bacterium, *Moraxella* sp., *in vitro*, and cause a marked reduction in the number of circulating haemocytes *in vivo* (Smith and Söderhäll, 1983), indicating that phenoloxidase and/or other components of the activating pathway function as opsonins in these animals. However, although the *in vitro* investigations revealed some degree of specificity in the cellular reaction to the glucans, equivalent events were not tested *in vivo*. The present study was therefore carried out to compare the *in vivo* haemocyte response of *C. maenas* to a range of structurally determined glycans, to assess the sensitivity of the cells to these carbohydrates and to determine the fate of those cells lost from the circulation.

MATERIALS AND METHODS

Animals

Specimens of *Carcinus maenas* were collected and kept as reported in Smith and Söderhäll (1983). Only healthy, intermoult, male crabs, measuring 60 ± 5 mm across the carapace, were used for experimental purposes. For crabs of this size, the haemolymph volume is *ca* 10–12 ml (Smith and Ratcliffe, 1980a).

Glycans

Laminaran (Calbiochem), a β 1,3-glucan, laminaran G and laminaran M, β 1,3-glucans terminated with glucose or mannitol respectively, were prepared and structurally determined as in Söderhäll and Unestam (1979). Curdlan, another β 1,3-glucan, was donated by Dr E. Reese, USA, while dextran T20, T40 and T2000 (Pharmacia), hyaluronic acid (Sigma), cellobiose (Pfanstiehl Labs), trehalose (Merck), glucose, mannose, mannitol and sorbitol (Merck) were obtained from commercial suppliers. All glycans were dissolved to the desired concentration in sterile *Carcinus* saline (Smith and Ratcliffe, 1978) as before (Smith and Söderhäll, 1983).

Injection and bleeding procedure

Injections (200 μl of the appropriate glycan or saline solution) were given through a membrane at the base of a pereiopod, as described in Smith and Ratcliffe (1980a). Haemolymph samples (250 μl) were withdrawn from a cheliped, as in Smith and Ratcliffe (1978) into 1.75 ml of citrate:EDTA buffer pH 4.6 (Söderhäll and Smith, 1983). To avoid the problem of the wide variation in the number of circulating haemocytes (THC) which often occurs between individual animals (Smith and Ratcliffe, 1980a), the effect of the glycans on the haemocyte number was determined for each crab by comparing the cell count before injection with the cell count after treatment. Preliminary experiments with uninoculated or saline injected *C. maenas* confirmed that, provided an interval of at least 1 hr elapsed between samplings, repeated bleeding had no significant effect on the THC. After injection, the crabs were incubated

in 8 μM-filtered sea-water (1.5 l/crab) at 12°C for 3 hr, the time previously found to show the maximum cellular response to the glucans (Smith and Söderhäll, 1983).

Cell counting

The haemocyte count in each haemolymph sample was determined with a Coulter Counter, model ZB (Coulter Electronics Ltd.), employing 0.22 μm-filtered artificial sea-water (Gerrard Biologicals), salinity 35‰, pH 7.4, as electrolyte.

Essentially, two haemocyte counting experiments were carried out; one to compare the cellular response to different glycans, and the other to monitor the dose response to injected laminaran. For the first experiment, groups of 5–10 crabs were given 200 μl doses of laminaran, laminaran G, laminaran M or curdlan (1 mg ml^{-1}) with further groups receiving 200 μl of dextran T20, T40 or T2000, hyaluronic acid, cellobiose, trehalose, glucose, mannose, mannitol or sorbitol (5 mg ml^{-1}). For the dose response, groups of 8–10 animals were inoculated with 200 μl of laminaran dissolved in sterile *Carcinus* saline at concentrations ranging from 5 mg to 5×10^{-4} mg ml^{-1}. Placebos received 200 μl of sterile saline.

The results were calculated as the percentage of haemocytes remaining in the circulation 3 hr after inoculation, and differences in these values between treatments were analysed with the Mann–Whitney *U*-test (one-tailed) (Siegel, 1956). The specified level of significance was $P \leq 0.01$.

Histology

To discover the fate of the haemocytes lost from circulation, gill filaments were excised from groups of three crabs inoculated 3, 6 or 24 hr previously with laminaran (5 mg ml^{-1}), glucose (5 mg ml^{-1}), dextran T40 (5 mg ml^{-1}) or sterile *Carcinus* saline. The gill pieces were then processed for wax histology as described in Smith and Ratcliffe (1980b).

RESULTS AND DISCUSSION

The effects of the various glycans on the THC of *C. maenas* are shown in Table 1. Laminaran, laminaran G, laminaran M and curdlan (1 mg ml^{-1}) all caused a significant decline in the number of circulating haemocytes after 3 hr, leaving only 58, 57, 51 and 27% of the cells in the haemolymph, respectively ($P > 0.05$ for each glucan compared to the saline injected controls). Similar reductions in the cell count did not occur in crabs treated with dextran T20, T40, T2000, hyaluronic acid, cellobiose, trehalose, glucose, mannose, mannitol or sorbitol, even at the higher dose of 5 mg ml^{-1}, and in these animals the number of cells remained close to, or slightly above, the number before treatment (Table 1). Saline injection, itself, tended to raise the THC (Table 1), although this increase was not significant ($P > 0.05$ compared to a group of nine untreated *C. maenas*, data not presented). In *C. maenas*, a decrease in the haemocyte number has previously been found to be induced by experimental infection with bacteria (Smith and Ratcliffe, 1980a), and this disappearance of haemocytes from the circulation usually signifies that a cellular defence reaction has taken place (Smith and Ratcliffe, 1980b). Thus, as a reduction in the THC was obtained in the present investigation only with the β (→3)-D-glucans and not with the other polysaccharides tested, it appears that *C. maenas* is capable of discriminating between different types of soluble molecules, and that the glucans alone triggered a defence-like haemocytic response *in vivo*. Unfortunately, it is impossible to state unequivocally that this cellular reaction is dependent only upon the β-1,3 linkage of the carbohydrates as a relatively restricted range of glycans were tested. Chitin and cellulose, examples of glucans with $\beta(1\rightarrow3;\ 1\rightarrow4)$ and $\beta(1\rightarrow4)$ linkages respectively, could not be used because of their insolubility in *Carcinus* saline. However, our previous *in vitro* analyses have shown that these sugars, unlike laminaran, are ineffective in promoting phagocytosis of bacteria (Smith and Söderhäll, 1983), so some degree of specificity does appear to occur in the cellular responses, and, more

Table 1. Effect of different glycans on the total haemocyte count (THC) of *C. maenas*

Glycan*	Glycan structure	Glycan concentration (mg ml^{-1})	Glycan dose (mg/crab)	% Haemocytes† remaining in the circulation after 3 hr	N‡
Laminaran	(1→3)β-D-glucan	5	1.0	28 ± 14§	10
Laminaran	(1→3)β-D-glucan	1	0.2	58 ± 12	8
Laminaran G	(1→3)β-D-glucan terminated with glucose	1	0.2	57 ± 17	7
Laminaran M	(1→3)β-D-glucan terminated with mannitol	1	0.2	51 ± 12	7
Curdlan	(1→3)β-D-glucan	1	0.2	27 ± 15	7
Dextran T20	α-D-glucan	5	1.0	96 ± 17	8
Dextran T40	α-D-glucan	5	1.0	100 ± 16	10
Dextran T2000	α-D-glucan	5	1.0	98 ± 20	8
Hyaluronic acid	(1→4)β-D-glucoronic acid; (1→3)β-*N*-acetyl D-glucosamine	5	1.0	99 ± 21	5
Cellobiose	(1→4)β-D-glucose	5	1.0	109 ± 27	5
Trehalose	(1→1)α-D-glucose	5	1.0	91 ± 11	6
Glucose		5	1.0	121 ± 9	5
Mannose		5	1.0	132 ± 27	5
Mannitol		5	1.0	119 ± 31	5
Sorbitol		5	1.0	104 ± 18	5
Carcinus saline		—	—	120 ± 37	10

*Each glycan was dissolved in sterile *Carcinus saline*. The glucans were structurally determined as in Söderhäll and Unestam (1979).

†Calculated for each crab as the $\frac{\text{THC at 3 hr}}{\text{THC at 0 hr}} \times 100$.

‡Number of animals tested.

§Values given as means ± SD.

importantly, this is the first report for the invertebrates of an *in vivo* cellular defence reaction elicited exclusively by soluble molecules.

Amongst the glucans tested, curdlan was the most effective in diminishing the haemocyte count. Possibly, the comparatively weaker, but still significant, effect of laminaran, laminaran G and laminaran M on the haemocytes was due to the lower degree of polymerization (of *ca* 20) of these laminaran-glucans (Söderhäll, unpublished). Certainly, the dose–response curve (Fig. 1) illustrates that with the higher laminaran dose (5 mg ml^{-1}), only 28% of the cells remained in the circulation after 3 hr, a value similar to those obtained with 5 mg ml^{-1} laminaran G and laminaran M (data not shown). Thus, it appears that while the presence/absence of a reduced end group on the glucans is unimportant for eliciting cellular activity, the $\beta(1\rightarrow3)$ linkages are essential. Figure 1 also shows that the magnitude of the host response is proportional to the concentration of glucan injected, and that a significant reduction in the THC was still obtained with 5×10^{-4} mg ml^{-1} laminaran. Since this represents a dose of *ca* 10^{-7} g per crab, the haemocytes of *C. maenas* clearly display very great sensitivity to these non-self molecules and are, therefore, very efficient in recognizing even low levels of foreign material in the haemocoel.

Regarding the fate of the cells lost from the circulation, examination of the gill sections, taken from crabs inoculated 3 hr previously with 5 mg ml^{-1} laminaran, revealed the presence of several small haemocyte clumps in the blood sinuses (Fig. 2). Haemocyte clumps were also detected in the gill sections after 6 and 24 hr incubation, although at these times the clumps were usually larger (Fig. 3) or more compact. Cell clumps were never seen in the gills of the saline, glucose or dextran injected crabs, and in these animals, large numbers of free haemocytes were observed in the blood channels (Figs 4 and 5). Since the cell aggregates, formed in response to the β 1,3-glucans, closely resemble those observed previously in *C. maenas* following injection of bacteria (Smith and Ratcliffe, 1980b), their development is probably controlled by the same mechanism. In *C. maenas* and other crustaceans, the β 1,3-glucans trigger activation of prophenoloxidase within the haemocytes (Söderhäll and Unestam, 1979; Smith and Söderhäll, 1983) by specifically activating one or more serine proteases in an enzymic cascade (Söderhäll, 1981; Söderhäll, 1983). This activating pathway must, therefore, be involved in haemocyte clumping, and the present investigation offers support for the proposal by Söderhäll (1982) that the prophenoloxidase activating system constitutes a major recognition mechanism in arthropods. Corroborative evidence is also provided by recent work by Söderhäll *et al.* (1983) who report that *A. astacus* exhibits stronger *in vivo* encapsulation reactions towards fungal spores coated in crayfish haemocyte lysate than to fungal spores coated in plasma or buffer. Moreover, as in *C. maenas* and other crustaceans, the prophenoloxidase system is confined to the granular and semi-granular cells (Söderhäll and Smith, 1983) whilst the hyaline cells as well as the granular and semi-granular cells participate in nodule formation (Smith and Ratcliffe, 1980b); and *in vitro*, β 1,3-glucans induce degranulation of the granular cells (Smith and Söderhäll, 1983), we suggest that, *in vivo*, the glucans act initially on the granular cells, inducing them to release substances (presumably phenoloxidase) from the granules which then assist in activating the hyaline cells. At present, we have no evidence that the glucans stimulate the hyaline cells directly, and investigations are currently underway to

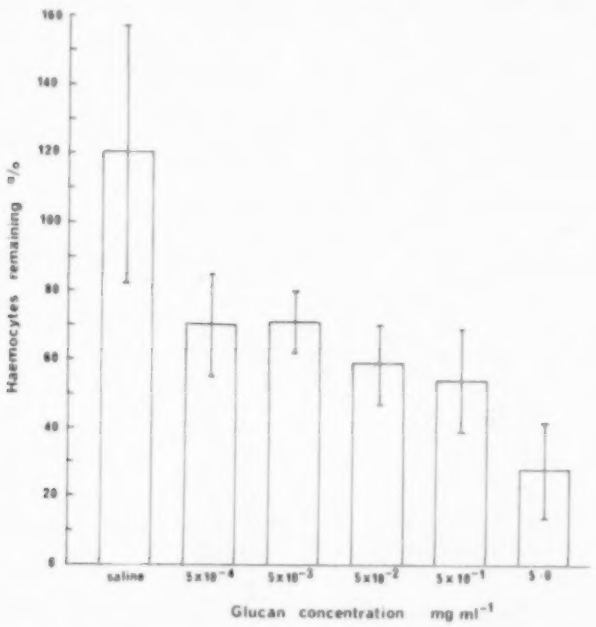

Fig. 1. Dose–response of *C. maenas* haemocytes to various concentrations of laminaran. Each point represents the mean of 8 or 10 animals ± SD.

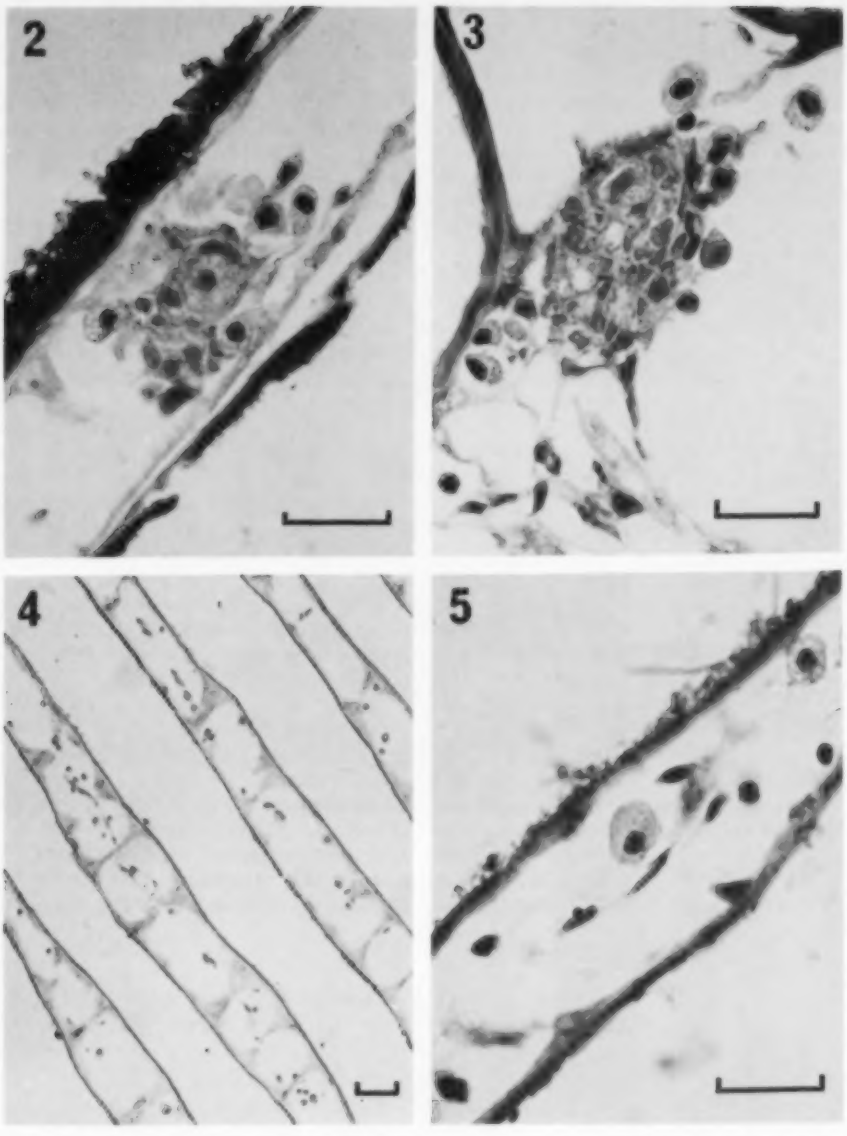

Fig. 2. Typical small haemocyte clump formed in the gills of *C. maenas* 3 hr after injection of laminaran (200 μl of 5 mg ml^{-1} solution). Giemsa stain. Scale bar = 100 μm.

Fig. 3. Typical haemocyte clump formed in the gills of *C. maenas* 6 hr after injection of laminaran (200 μl of 5 mg ml^{-1} solution). The cell clump is larger and more compact than the 3 hr clump. Giemsa stain. Scale bar = 100 μm.

Fig. 4. Gill filaments from a crab inoculated 3 hr previously with 200 μl of glucose (5 mg ml^{-1}). Several haemocytes can be seen circulating freely in the haemolymph with no evidence of cell clumping. Giemsa stain. Scale bar = 100 μm.

Fig. 5. Gill filament from a crab inoculated 24 hr previously with 200μl saline. As with the glucose or dextran T40 treated animals at this time there is still no evidence of cell clumping. Giemsa. Scale bar 100 μm.

elucidate the biochemical nature of any cell to cell communication involved in the cellular defences of crustaceans.

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HEAT RESISTANCE AND CONTRACTILE VACUOLAR ACTIVITY OF *PARAMECIUM CAUDATUM* ACCLIMATED TO DIFFERENT TEMPERATURES

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Abstract—1. *Paramecium* acclimated to 25°C was more heat resistant than 10°C-acclimated *Paramecium*.
2. Change in heat resistance after transfer to a new acclimation temperature was completed in 6–9 days.
3. The vacuolar frequency–temperature curve of 25°C-acclimated *Paramecium* shifted toward higher temperature.
4. Thus *Paramecium* is possessed of the ability of capacity adaptation as well as resistance adaptation to temperature.

INTRODUCTION

Temperature adaptation has been divided by the response of animals into two categories (Precht *et al.*, 1955): one is the adaptation to the lethal effect of temperature, "resistance adaptation" and the other is the adaptation in various rate functions at sublethal or physiological temperature, "capacity adaptation".

Phenomena of temperature adaptation in Paramecia have been summarized by Wichterman (1953) and Poljansky and Sukhanova (1967). It has been found that Paramecia have the ability to acclimate gradually to a high temperature (Jacobs, 1919), to cold (Efimoff and Efimoff, 1924) and to subzero temperatures (Wolfson, 1935). Mendelssohn (1902) noted that optimum temperature rose in Paramecia kept at a higher temperature for some hours. Jollos (1913, 1914, 1921) found that the thermal death point could be raised 1–3°C in long-continued experiments for acclimatization. Poljansky (1957, 1959) showed that the thermoresistance examined in clone material of *Paramecium caudatum* was under the direct influence of the environmental temperature. However, these are studies principally on "resistance adaptation" and phenomena comparable to "capacity adaptation" in Paramecia have been not found. Investigation of the capacity adaptation in various physiological functions is important to elucidate the mechanism of physiological regulation against change in environmental temperature.

This paper is the report for the time course of changing heat resistance and for "capacity adaptation" in pulsation rate of the contractile vacuole in *Paramecium caudatum* acclimated to two different temperatures.

MATERIALS AND METHODS

The Paramecia used in the present study were three clones (A, B and C) originated from a strain of the *Paramecium caudatum* which has been cultured for several years under constant conditions in our laboratory. Each clone was divided into two lines one of which was cultured at 10°C (cold acclimation) and the other at 25°C (warm acclimation). This acclimation culture was continued above three months before determination of heat resistance and vacuolar activity.

The heat resistance was determined by the survival ratio after exposure to a given high temperature for 5–40 min. Small test tubes containing 0.5 ml of suspension of the Paramecia were immersed into a warm bath controlled at a constant temperature and taken out successively from the bath after 5, 10, 20, 30 and 40 min for count of the survival ratio by the following procedure. The suspension of Paramecia was dropped on a glass slide with viscous 6% methylcellulose solution and survival or death was microscopically observed. The survival ratio per 250–300 Paramecia was calculated from the random observation of several fields.

The activity of the contractile vacuole was microscopically observed under control of temperature using a glass vessel connected to a water circulation system. A known volume of the suspension of Paramecia kept at a test temperature was dropped into the glass vessel and 0.1% $NiSO_4$ solution of 1/10 volume of the suspension was added. The time taken for two contractions of the anterior contractile vacuole was recorded. This was initiated by measurement at the acclimation temperature to avoid the shocking effect of an abrupt temperature change. Before the succeeding measurement, the temperature of the system was raised or lowered by 3°C at a rate of 0.5°C per min and so measurement was continued from the acclimation temperature to the highest or to the lowest temperature.

The effect of the medium concentration on the vacuolar frequency at various temperatures was determined using media of different concentrations prepared by addition of sucrose.

RESULTS

Figure 1 shows the heat resistance of three clones of *Paramecium caudatum* in survival ratio at three high temperatures. The Paramecia acclimated to 25°C survived after 40 min exposure to 36.5 and 38°C in all clones, while 10°C-acclimated Paramecia were subjected to lethal effect after 5 min at 36.5°C. The effect of 41°C was lethal on either of 10°C- and 25°C-acclimated Paramecia, but median survival time of the former was obviously shorter than that of the latter in any clone. The Paramecia acclimated to 25°C

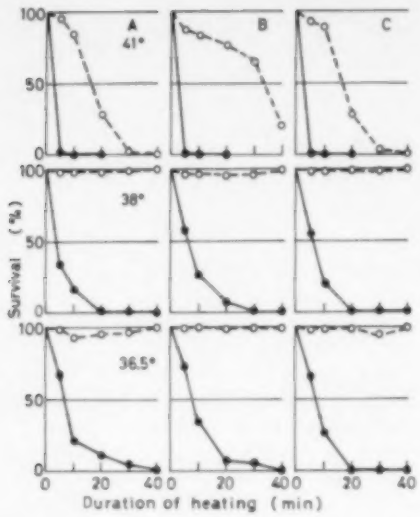

Fig. 1. Survival curves of three clones, A (left), B (middle) and C (right), of the *Paramecium caudatum* acclimated to 10°C (solid circles) and to 25°C (empty circles). These were measured at 41°C (top), 38°C (middle) and 36.5°C (bottom). Each point is the mean value for 10 Paramecia.

are distinctly heat resistant in comparison with 10°C-acclimated Paramecia.

Figure 2 shows the time course of change in heat resistance after transfer from one to another temperature of 10 and 25°C. Conditions of heat treatment were at 41°C for 10 min, so as to bring on 100% survival in 25°C-acclimated Paramecia and zero in 10°C-acclimated ones. When the Paramecia were transferred from 10 to 25°C, the heat resistance comparable to 25°C-acclimated Paramecia was gained after about seven days and in the opposite case the heat resistance declined to the level of 10°C-acclimated Paramecia within 10 days in any clones.

Figure 3 shows activity of the anterior contractile vacuole at various temperatures in mean frequency per min for 10 Paramecia. The vacuolar frequency generally increased with rising temperature up to a high temperature above which the frequency decreased. The frequency of 25°C-acclimated Paramecia was higher above about 17°C in clone A, 14.5°C in clone B and 15°C in clone C than that of 10°C-acclimated Paramecia and below these temperatures this relation was reversed. These facts indicate that environmental temperature affects temperature dependence of the vacuolar frequency and the temperature–frequency curve of the warm acclimated Paramecia shifts toward higher temperature. The vacuolar frequency in clone C was generally lowest among the three clones, especially at higher temperatures. To confirm the differences between clones or between temperature acclimation lines, statistical analysis of variance was tried for values measured at 9, 15, 24 and 30°C. These results are given in Table 1. The difference between clones was not significant at any of the four temperatures. The difference between 10°C- and 25°C-acclimated lines was significant at 9, 24 and 30°C, but not significant at 15°C. The interaction between clones and acclimation lines was non-significant at any temperature.

The effect of the medium concentration on the vacuolar frequency was determined at 15, 20, 25 and 30°C. Results are given in Fig. 4 and Table 2. Each point in Fig. 4 is the mean vacuolar frequency for 10 Paramecia at different sucrose concentrations equivalent to 0–6% sea-water. The vacuolar frequency seemed to decrease linearly with increasing concentration, so regression lines calculated were added in Fig. 4. Table 2 shows the test of linearity and the difference between temperature acclimation lines in the regression lines. In many cases, especially in all at 20 and

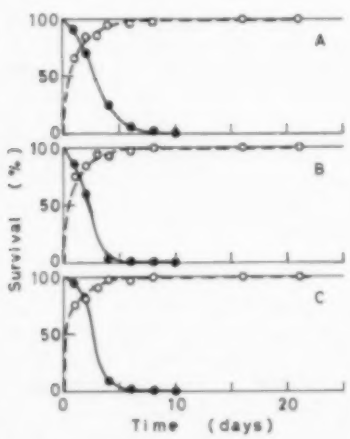

Fig. 2. Time course of change in the heat resistance of the *Paramecium caudatum* transferred from 10 to 25°C (empty circles) and in reverse (solid circles). Top: clone A, middle: clone B, bottom: clone C.

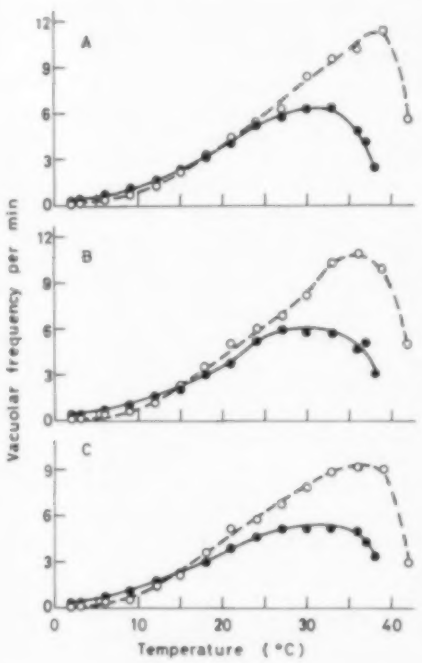

Fig. 3. Temperature dependence of the anterior contractile vacuole of the *Paramecium caudatum* acclimated to 10°C (solid circles) and to 25°C (empty circles) of three clones, A (top), B (middle) and C (bottom).

Table 1. Analysis of variance of the vacuolar frequency in three clones of *Paramecium caudatum* acclimated to 10 and 25°C. *C*: between clones; *T*: between acclimation temperatures; *C* × *T*: clone-acclimation temperature interaction; *E*: within subclones

Test temp. (°C)	Source of variation	Degrees of freedom	Sum of squares	Variance ratio (*F*)	Levels of significance
9	*C*	2	0.665	0.388	$0.05 < P$
	T	1	141.681	165.065	$P < 0.001$
	C × *T*	2	1.241	0.723	$0.05 < P$
	E	54	0.858		
15	*C*	2	9.847	1.011	$0.05 < P$
	T	1	0.140	0.029	$0.05 < P$
	C × *T*	2	2.500	0.257	$0.05 < P$
	E	54	263.032		
24	*C*	2	104.094	4.904	$0.01 < P < 0.05$
	T	1	271.363	25.567	$P < 0.001$
	C × *T*	2	53.576	2.524	$0.05 < P$
	E	54	573.150		
30	*C*	2	316.756	4.579	$0.01 < P < 0.05$
	T	1	3796.922	109.779	$P < 0.001$
	C × *T*	2	47.341	0.684	$0.05 < P$
	E	54	1867.703		

25°C, the frequency–concentration relation was regarded as linear. The regression coefficient (*b*), or slope of regression line, increased with rising temperature in any clone. There was no significant difference in the slopes of the regression lines between 10°C- and 25°C-acclimated Paramecia in any case except for clone C at 30°C. Mean frequency ($\bar{y}$), or level of regression line, increased too with rising temperature, although the difference between temperature acclimation lines was significant at 25 and 30°C, but not at 15 and 20°C. This is reasonable from the fact that the vacuolar frequency of the cold acclimated Paramecia was higher below about 15°C and lower above it than that of the warm acclimated ones.

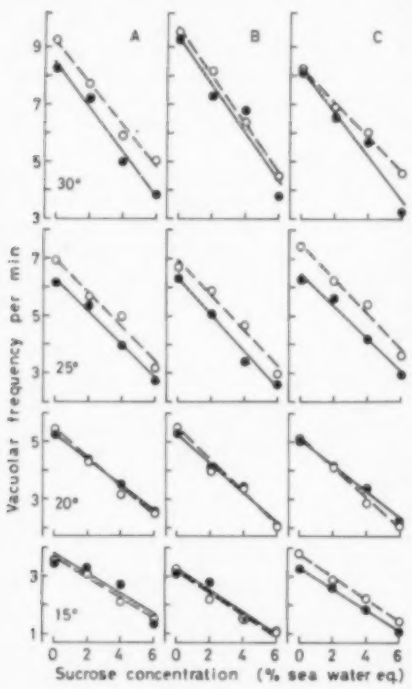

Fig. 4. Regression lines of the vacuolar frequency on the sucrose concentration at 30, 25, 20 and 15°C from top to bottom in the *Paramecium caudatum* acclimated to 10°C (solid circles and solid lines) and to 25°C (empty circles and broken lines). Left: clone A, middle: clone B, right: clone C.

DISCUSSION

Heat resistance of the *Paramecium caudatum* of three clones changed in the parallel direction with acclimation temperature (Fig. 1) as found in other previous investigations (Wichterman, 1953; Poljansky and Sukhanova, 1967).

Jollos (1913, 1914, 1921) found the following fact in one clone of *Paramecium aurelia* subjected to 31°C for some years, that the heat tolerance increase during long time culture at the high temperature lasted in some cases up to six months after removal from the high temperature, but the tolerance finally disappeared. According to Poljansky (1957) change in thermoresistance of *Paramecium caudatum* proceeded very quickly to the maximum, sometimes within several hours after transfer to a warm environment. In the present experiment, the rate of change in the heat resistance was not as high as Poljansky described, but the heat resistance gained did not last much longer than *Paramecium aurelia* cited above. Both gain and loss of the heat resistance reached the maximum in about a week after transfer from one to another temperature (Fig. 2). It has been found in a teleost, guppy, that gain of the heat resistance was generally faster than loss of it (Doudoroff, 1942), while the rate of the gain and loss was dependent on the temperature of the second acclimation as well as on the width of the temperature range between the first and second acclimation (Tsukuda, 1960). In *Paramecium* also the rate of change in the heat resistance could vary with ambient temperature.

Since *Paramecium* divided once every two days at 10°C and three times a day at 25°C under the conditions in our laboratory, the rate of change in the heat resistance may be independent of the fission rate.

Table 2. Linearity of regression lines of vacuolar frequency of medium concentration and test of differences between acclimation temperature (AT) in *Paramecium caudatum*

TT* (°C)	Clone	AT (°C)	Test of linearity (Levels of sig.)	Regression line $y = \bar{y} - b(x - \bar{x})$, $\bar{x} = 3$ $\bar{y}$	b	Difference between acclimation temperature (Levels of sig.) $\bar{y}$	b
15	A	10	$P < 0.001$	2.74	−0.362	$0.05 < P$	$0.05 < P$
		25	$0.05 < P$	2.62	−0.353		
	B	10	$0.001 < P < 0.01$	2.12	−0.380	$0.05 < P$	$0.05 < P$
		25	$0.001 < P < 0.01$	2.05	−0.381		
	C	10	$0.05 < P$	2.22	−0.362	$0.05 < P$	$0.05 < P$
		25	$0.05 < P$	2.59	−0.388		
20	A	10	$0.05 < P$	3.93	−0.437	$0.05 < P$	$0.05 < P$
		25	$0.05 < P$	3.88	−0.491		
	B	10	$0.05 < P$	3.73	−0.501	$0.05 < P$	$0.05 < P$
		25	$0.05 < P$	3.78	−0.561		
	C	10	$0.05 < P$	3.72	−0.459	$0.05 < P$	$0.05 < P$
		25	$0.05 < P$	3.55	−0.527		
25	A	10	$0.05 < P$	4.57	−0.585	$P < 0.001$	$0.05 < P$
		25	$0.05 < P$	5.20	−0.605		
	B	10	$0.05 < P$	4.37	−0.636	$P < 0.001$	$0.05 < P$
		25	$0.05 < P$	5.09	−0.625		
	C	10	$0.05 < P$	4.79	−0.565	$P < 0.001$	$0.05 < P$
		25	$0.05 < P$	5.70	−0.608		
30	A	10	$0.01 < P < 0.05$	6.11	−0.784	$P < 0.001$	$0.05 < P$
		25	$0.05 < P$	7.01	−0.724		
	B	10	$P < 0.001$	6.80	−0.844	$0.01 < P < 0.05$	$0.05 < P$
		25	$0.05 < P$	7.15	−0.839		
	C	10	$0.001 < P < 0.01$	5.91	−0.791	$P < 0.001$	$0.001 < P < 0.01$
		25	$0.05 < P$	6.47	−0.593		

*Temperature of test.

It has been shown that there were some differences in pulsation rate between the anterior and posterior contractile vacuole (Child, 1914; Unger, 1926; Frisch, 1937) and that the anterior vacuole pulsated more rapidly than the posterior one in *Paramecium caudatum* (Unger, 1926). In the present experiment the pulsation of only the anterior vacuole was observed.

The vacuolar frequency increases with rising temperature in some protozoa (Cole, 1925; Gaw, 1936; Kitching, 1954). The vacuolar frequency of the *Paramecium caudatum* in the present experiment also increased with rising temperature up to a temperature above which the frequency decreased. This conforms to general rate–temperature relation in responses of poikilotherms. The rate–temperature curves of the warm acclimated Paramecia shifted toward higher temperature as compared with the curves of the cold acclimated Paramecia (Fig. 3) as found in many rate functions of thermally acclimated poikilotherms (Tsukuda and Ohsawa, 1959; Tsukuda, 1961; Takamatsu and Tsukuda, 1977). This suggests the adaptive effect of environmental temperatures. However, it seems different from the capacity adaptation in various multicellular poikilotherms at the point that the rate–temperature curves of the warm and cold acclimated Paramecia intersected at relatively lower temperatures.

Herfs (1922) showed that the vacuolar frequency of *Paramecium caudatum* linearly decreased with increasing concentration of sodium chloride from 0 to 0.75% at about 20°C. In the present experiment also, the vacuolar frequency–concentration relation was linear, especially at 20 and 25°C (Table 2). This indicates that the water volume discharged from contractile vacuoles is equal to water inflow. Discharged volume is the product of vacuolar frequency and volume, while inflow is proportional to permeability and surface area of a cell and concentration gradient between intracellular and extracellular fluids. If the vacuolar volume, surface area and intracellular concentration are constant, a linear relation between the vacuolar frequency and the extracellular concentration suggests that the permeability is constant. Between the cold and the warm acclimated Paramecia there was no significant difference in slopes of regression lines (regression coefficient) in many cases, while there was a significant difference in levels of regression lines (mean frequency) (Table 2). These facts suggest that permeability of the Paramecia is not variable, but intracellular osmotic concentration varies in relation to environmental temperature.

Recently, effects of temperature on the behavioral responses have been found by Tawada and Oosawa (1972), Nakaoka and Oosawa (1977) and Kawakubo and Tsuchiya (1981). These are interesting problems for further study on temperature adaptation in Paramecia.

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FUNCTIONING OF CATFISH ELECTRORECEPTORS: BURSTING DISCHARGE PATTERN OF *KRYPTOPTERUS* ELECTRORECEPTORS ELICITED BY MICROELECTRODE IMPALEMENT

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Abstract—1. The time course of the spontaneous discharge of *Kryptopterus* ampullary electroreceptors may change from regular to bursting upon impalement by a glass microelectrode.

2. The burst duration is very constant relative to the interburst interval, during spontaneous fluctuations as well as in response to low-frequency stimulation.

3. Implications for the current model of the functioning of catfish electroreceptors are discussed.

INTRODUCTION

The electroreceptors of the transparent catfish *Kryptopterus bicirrhis* are of the ampullary type (Wachtel and Szamier, 1969) and are sensitive to low-frequency electric currents (Bennett, 1971; Roth, 1973, 1978). Although the course of the transmission from electric current in the water to afferent nerve impulses is not yet fully understood, the following processes are likely to occur when a receptor is stimulated by an anodal, i.e. inward current. The stimulus current flows through the apical membranes of the receptor cells, which results in a slight depolarization of the intracellular potential. This depolarization (probably) causes an inflow of calcium ions which, in turn, enhance the release of an exciting transmitter substance. The postsynaptic membrane is depolarized by the increased transmitter flux, which results in a higher impulse rate at the spike generation site (Bennett and Clusin, 1979; Teeter and Bennett, 1981).

Due to stray fields, the impulses can be recorded from the lumen of the receptors, as well as from the receptor cells (Bennett, 1971). The former method, being non-invasive, ensures undisturbed functioning of the receptor, whereas the latter permits measurement of the membrane potential of the receptor cells. Contrary to the situation in other types of electroreceptors, the apical membranes of ampullary electroreceptors of freshwater catfish appear to be electrically non-excitable (the reader is referred to Bennett and Clusin, 1979, for a full discussion of this subject).

However, following impalement of a sensory cell with a glass microelectrode (Fig. 1) the nerve activity often changes from fairly regular firing into a conspicuous bursting discharge pattern. This usually lasts for a few minutes, after which the regular discharge pattern is re-established. The occurrence of bursts can usually be prevented by the use of relatively high-impedance micropipettes (30–50 MΩ) and by very careful electrode insertion using a high-magnification microscope. Nevertheless, we decided for a further examination of the bursting discharge pattern for the following reasons.

First, the easy to obtain and reproducible sudden change to a bursting discharge pattern is not to be expected in sensory organs, their main function being reliable and continuous transduction of external information into a frequency-coded nerve signal.

Secondly, understanding the bursting mechanism might give clues about normal receptor functioning. Besides recording and analysing the bursting pattern, we tried to influence bursting electroreceptors by electrical stimulation. Implications of the results for the current model of the functioning of catfish electroreceptors are discussed.

MATERIALS AND METHODS

Transparent catfish (*Kryptopterus bicirrhis*), about 4 cm long, were placed in a shallow dish, filled with recirculating aerated water of 25 ± 1°C after immobilization with Flaxédil (gallamine; Specia, Paris). The same water was supplied to the fish through a polythene cannula into the oral cavity. The dish was mounted on the stage of a microscope fitted with water immersion objective lenses, allowing observation of the electroreceptors with a high magnification, using transmitted light. The immobilization of the fish usually lasted for about 3 hr, after which the fish were returned to the stock tank.

We used glass micropipette recording electrodes filled with 3.0 M KCl (impedance 5-20 MΩ). For impalement, the microelectrode was manoeuvred through the porus of the receptor in such a way that a receptor cell could be penetrated by a slight, axial movement of the manipulator. A reference electrode having a larger tip diameter and filled with 0.1 M KCl was placed in the water near the fish. The differential signal was amplified (bandwidth d.c.–3 kHz) and stored on magnetic tape.

Interspike intervals were measured with an electronic counter and fed into a microcomputer. As the interburst intervals were substantially longer than any interspike interval within a burst, the spike train was analysed by segregating intervals into two classes. Thereafter, the burst duration, the mean frequency during a burst (the number of spikes in a burst divided by the burst duration) and the

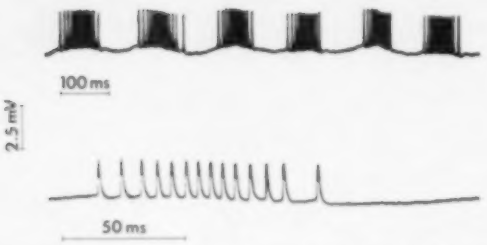

Fig. 1. Example of a bursting spike brain from an electroreceptor. Upper trace: six consecutive bursts on a time scale that precludes resolution of all individual spikes in a burst. Lower trace: one burst on a faster time scale.

"period" (sum of burst duration and the next interburst interval) were calculated. These parameters were plotted as a function of the burst number, together with the interburst interval.

To investigate the role of the synapses in the bursting mechanism, both bursting and normal functioning electroreceptors were stimulated electrically. Stimulus current was applied by a fork-shaped silver wire, placed near the recording electrode. The effects of a low frequency (0.3 Hz) sinusoidal stimulus current and of anodal and cathodal direct current on the above-mentioned parameters were observed. The influence of a strong cathodal current on the sensitivity of the normal functioning (non-bursting) electroreceptor was investigated by superimposing an alternating current (5 Hz) on the d.c.-steps, while recording from the lumen (cf. Bretschneider *et al.*, 1980, for the effects of cathodal current on electroreceptors of *Ictalurus nebulosus*).

RESULTS

Burst characterization

Upon penetration, the potential measured by the microelectrode usually jumped to between −20 and −30 mV, followed by a slow decrease to a value between zero and −10 mV. Bursting usually followed impalement within a few seconds. In all, 41 receptors (of 11 fish) were impaled, followed by bursting in 36 cases. In most cases, bursting lasted for several minutes, allowing characterization of both the spontaneous behaviour and the response to electrical stimulation. An example of the spontaneous bursting pattern is shown in Fig. 1. Figure 2 shows the time course of the variables burst duration, interburst interval, period and mean spike frequency in a burst for a series of 130 consecutive bursts (*ca.* 25 sec). Although the burst duration varied from 40 to 90 msec between experiments, the burst duration within a series is fairly constant, whereas the interburst interval is not. It should be mentioned, however, that a few deviating bursts occurred in the transitions from continuous firing to bursting and

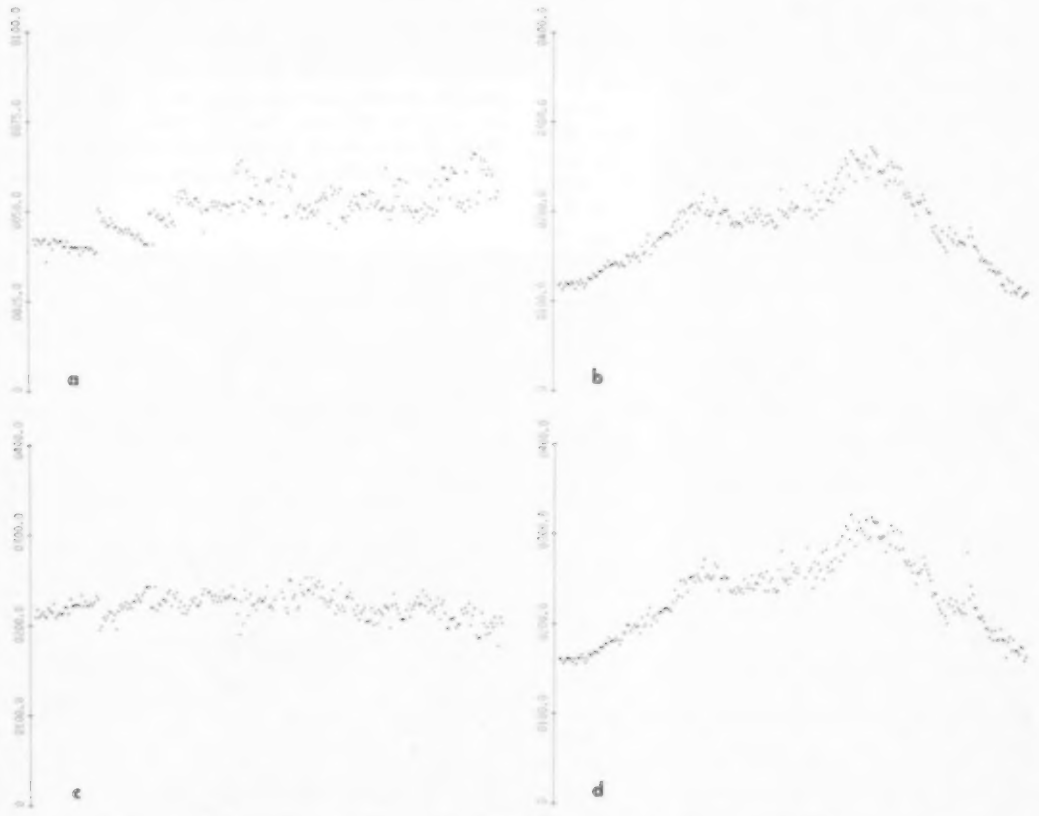

Fig. 2. Example of spontaneous variation of the variables (a) burst duration, (b) interburst interval, (c) mean spike frequency in burst, and (d) burst period. Horizontal axis: serial number of burst; vertical axis: time in msec in (a), (b) and (d) frequency in spikes/sec in (c).

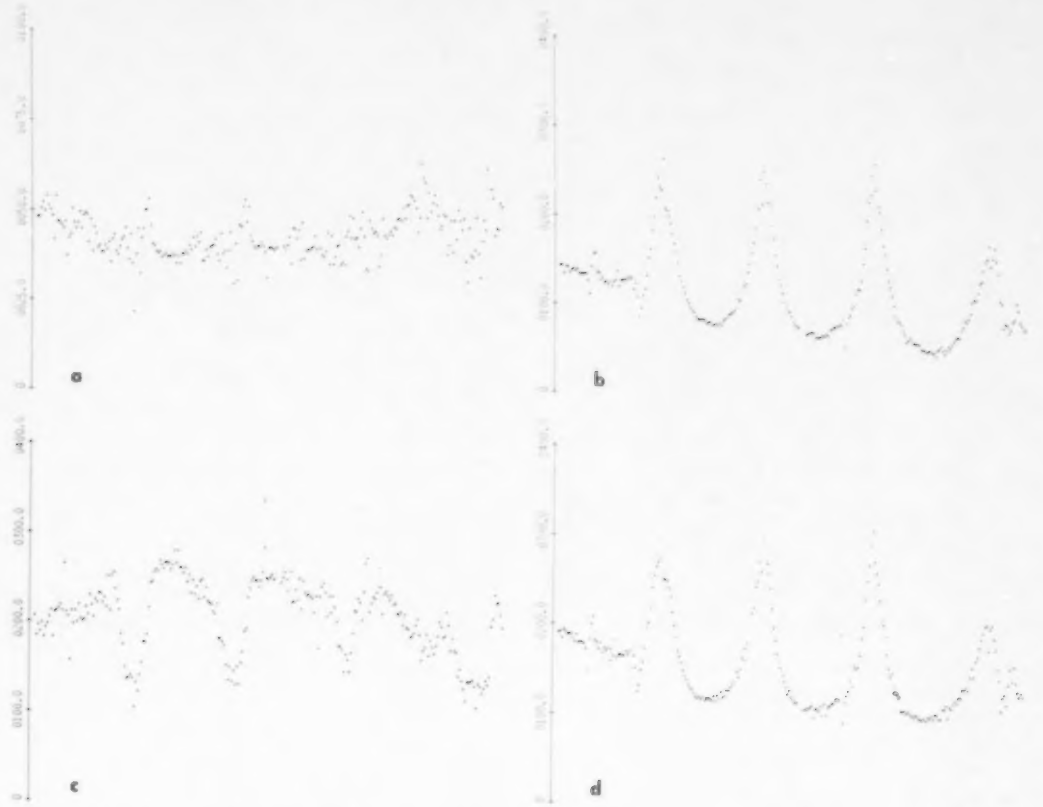

Fig. 3. Example of modulation of burst variables by a 0.3 Hz sinusoidal stimulus current. Meaning of letters (a)–(d) and calibration of axes as in Fig. 2.

vice versa. These are not included in the figures. Not depicted is the number of spikes per burst, which varied between 7 and 15 between experiments. In Fig. 2, discontinuities in burst duration and mean spike frequency in burst can be seen at each addition of one spike per burst.

Low-frequency stimulation

When an electroreceptor is stimulated with a sinusoidal current (0.3 Hz, 100 nA/cm^2) during bursting, the difference in variation between the burst duration and the interburst interval becomes even more pronounced. The low-frequency current "modulates" the burst parameters. The modulation of the interburst interval is larger than the modulation of the burst duration (Fig. 3). The "instantaneous frequency" (reciprocal of interburst interval) responds linearly (i.e. sinusoidally) to the sinusoidal stimulus current.

Direct current stimulation

The effects of stimulation with direct current on the bursting discharge pattern can be described as follows. The influence of relatively weak (<250 nA/cm^2) anodal and cathodal d.c.-steps was small: after an initial increase or decrease of the firing frequency, the original bursting discharge pattern was re-established in a few seconds. Stimulation with a strong anodal (exciting) direct current (250–500 nA/cm^2) changed the discharge pattern into regular high-frequency firing. In most cases the bursting pattern reappeared within one minute. The bursting responded different to a strong cathodal (inhibiting) direct current. After an initial silent period of a few seconds, bursting reappeared with a longer period. In a few minutes the burst frequency returned to the normal (unstimulated) level.

Normal, i.e. non-impaled, electroreceptors responded to a cathodal current step in the same way as electroreceptors of *Ictalurus* (Bretschneider *et al.*, 1980): the spike rate is zero for a few seconds, after which a low, regular spike rate is established. During this period, the receptor is insensitive for an added alternating current. Normal spike rate and a.c.-sensitivity return slowly in about 10 min.

DISCUSSION

The results can be summarized as follows: upon impalement of a receptor cell with a glass microelectrode, the normally regular spike train often starts bursting. The duration of the bursts is rather constant; about 60 msec. The interburst intervals are, on the contrary, of variable duration, showing both spontaneous variation and response to electrical stimulation.

The oscillatory membrane potential excursions that presumably underlie the intermittent spike gen-

eration may arise in either pre- or postsynaptical parts of the receptor. If the time course of the spike rate is taken as an approximate measure of the hypothetical membrane potential variation, the burst cycle resembles a slow action potential. Similar, slow action potentials, often caused by a calcium influx mechanism and called calcium spikes for short, have been found elsewhere, e.g. in the ampullary electroreceptors of skates. In this receptor type, the spike-like oscillations are found at the apical membranes of the receptor cells, and can be recorded from the lumen of the ampullae (Obara and Bennett, 1972). This electrical excitability is thought to be an explanation of the high electrosensitivity of skate electroreceptors (cf. Bennett and Clusin, 1979). The mode of operation of catfish electroreceptors is, however, thought to be different. Intracellular measurements have shown, so far, only absence of electrical excitability of the apical membrane. This was stated by Bennett (1971) and confirmed by our own measurements. Added to the observation of post-synaptic potentials of normal, i.e. large size in the marine catfish *Plotosus*, this leaves the synapses of catfish electroreceptors as the most electrosensitive component in the transduction chain (Bennett and Clusin, 1979).

Although bursting in *Kryptopterus* receptors is initiated by impalement of a receptor cell, we do not expect the oscillation to arise presynaptically for the following reasons. In the first place, no voltage excursions large enough to be responsible for the observed, vigorous spike frequency modulation, are recorded by the intracellular electrode that initiated the bursting. Secondly, the bursting could not be suppressed by a negative current of several microampere, whereas such a current does abolish the normal functioning of intact receptors. The presynaptical disturbance that starts the bursting might be a depolarization caused by electrode leakage. This might be indicated by the rather low intracellular potentials we found. Such low membrane potentials have been found in several types of small, cultured cells and have been ascribed to a leakage resistance formed by the hydration mantle of the glass micropipette (Lassen *et al.*, 1971; Ince *et al.*, 1983). The surface area of the receptor cells of *Kryptopterus* electroreceptors is of the same order of magnitude (*ca.* 500 μm^2).

If the bursting can be evoked by a depolarization of only one of the 20–25 receptor cells of a *Kryptopterus* electroreceptor, the postsynaptic potential has to be rather close to an instability. This suggests then, that besides the synaptical transduction, the electrical properties of the postsynaptic membranes are a factor enhancing the overall electrical sensitivity of catfish electroreceptors.

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IRON CONTENT IN STARLINGS, *STURNUS VULGARIS* L.

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Abstract—1. Several groups of starlings (*Sturnus vulgaris* L.), captured in north-eastern Spain (Delta del Ebro, Tarragona) during winter migration (November–January) were studied.

2. Hematological values (hematocrit, hemoglobin concentration and reticulocytes), iron concentrations and the weights of different organs (feathers, liver, spleen, pectoral muscle, heart, intestine, plasma) and the whole body, were analysed and compared to values obtained from non-laying female chickens and quails.

3. The strong flying habits of this species can be seen in the heart size (1.2% body wt), breast muscle (17.8% body wt), hematocrit (44–47%) and hemoglobin concentration (14.5–16.2 g%).

4. The iron content in the organs was in general 2–10 times higher than in chickens or quails, especially in the liver (1310–3300 μg Fe/g wet) and feathers (380–780 μg/g wet).

5. The iron concentration per body weight ranged from 153 to 185 ppm, which was 2–4 times higher than the values for other birds or mammals.

INTRODUCTION

We began a study on iron levels in the different organs and in the whole bird with domestic fowl; chicken and quail (unpublished data). At the same time, iron (^{59}Fe) distribution was analysed in pigeons (Ramis and Planas, 1978). Therefore, we were interested in extending our studies to include a wild species, such as the starling. This species is a good flapping flyer and a winter resident in our country. The starling has been used as a monitor species for metal contamination (Martin, 1972) and also Osborn (1979) has analysed seasonal variations in iron liver in resident British specimens. In *Sturnus roseus*, Pilo (1979) has studied the iron content of the liver and pectoral muscle, during the post- and pre-migratory periods in India. However, we have not found any other data on iron distribution for *Sturnus vulgaris*.

We analysed the iron content in starlings, caught in Delta del Ebro (Tarragona) during their winter stay. For this study, we determined the whole iron content and the distribution of iron in the main organs and compared the results to data obtained in our laboratory from a non-flying bird, the chicken, and from a poor flyer, the quail.

MATERIALS AND METHODS

The study was done with starlings *Sturnus vulgaris* L. The birds were caught in the Delta del Ebro (Tarragona) with Japanese nets. Dates of capture are listed chronologically: (A) 16 November 1980, (B) 29 January 1981, (C) 4 November 1981, (D) 15 December 1981 (capture always took place at dusk). In this region, the starlings arrive from central Europe in October, and return in February. Of course, weather conditions modify the exact dates of arrival and departure. The different groups analysed were representative of (a) those just arrived (groups A and C), (b) winter residents (group D) and (c) those preparing for migration (group B).

The birds were transported to our laboratory, 10–20 hr after capture. Groups of five or six were immediately bled to death by heart puncture, with a heparinized needle (gauge 30/8) joined to a catheter, the end of which was introduced into a plastic tube. A vibrating device, attached to the tube, facilitated the blood flow and avoided coagulation of the sample. The birds were weighed alive, using an Ohaus balance. They were plucked, and body and feathers were weighed separately.

The remaining birds were kept in large wire cages (50 × 100 and 120 cm high) which were covered with brown paper, to avoid excitement. The birds were fed commercial chicken food and apples.

The hematocrit was determined in microhematocrit capillary tubes and centrifuged at 11,000 rpm for 6 min in Haemofuge (Heraeus, F.R.G.). The hemoglobin concentration, in 0.01 ml blood, was measured with Drabkin's reagent, and standards were obtained from Hycel. The erythrocyte number was determined using a Thoma's chamber.

The specimens used for iron content evaluations in different organs, were weighed and later bled in the same way as above. The featherless body and the feathers were weighed separately in a Mettler balance, accurate to 0.01 g. The other organs studied (heart, liver, pectoral muscle, breast, spleen, femur bone marrow and intestines) were also weighed in the Mettler balance.

Iron determination of the tissues was done by atomic absorption spectroscopy (AAS), according to the Theron *et al.* (1973) technique, using a Pye Unicam SP-1900 from the "Servicio de Espectroscopía" at this University. The samples were washed three times in saline, before acid digestion (sulfuric and nitric acids, 1:3). Either the whole organ (spleen, heart and bone marrow) or a 1 g piece of tissue (liver, pectoral muscle and feathers) was used.

For an iron content evaluation of the whole bird, the specimens were killed with ether. Then they were plucked and weighed again in the Ohaus balance. The featherless

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Table 1. Body and organ weights in grams and also in percentages of the total body weight from various groups of starlings. Mean ± SE

Organs	Number specimens	Weights: Absolute weight (g)	Weights: Relative weight (% body wt)
Body weight	43	64.72 ± 1.61	—
Feathers	20	5.21 ± 0.13	8.0 ± 0.3
Liver	29	1.59 ± 0.05	2.4 ± 0.1
Pectoral muscle	24	11.50 ± 0.34	17.8 ± 0.5
Spleen	27	0.04 ± 0.002	0.1 ± 0.01
Bone marrow	25	0.25 ± 0.01	0.4 ± 0.1
Intestine			
Duodenum	27	1.12 ± 0.07	1.5 ± 0.1
Jejunum	27	0.70 ± 0.04	1.1 ± 0.1
Ileum	27	0.48 ± 0.04	0.7 ± 0.1
Heart	24	0.76 ± 0.02	1.2 ± 0.09

body was dehydrated in a oven at 110 C for 48 hr, and later incinerated in a 1300 W electric furnace for 24 hr. The ashes were dissolved in an acid-digestion mixture, the same as used for the 1 g sample of feathers, and iron content was determined by AAS.

Plasma iron was evaluated by the beta-phenantroline method, recommended by International Committee (1972). The total iron binding capacity was determined following Ramsay's method (1957). Reticulocytes were identified with a cresyl violet stain (Lucas and Jamroz, 1961).

The data were statistically analysed by Kruskall-Wallis' non-parametric test and, in some cases, by the Mann–Whitney test.

RESULTS

Whole body weights and those of the different organs, are shown in Table 1. The data for each capture group were independently analysed and comparisons among them were established. As no variations in weight were statistically observed, because of the dates of capture, we here present only the average of all of them together.

Blood parameters, such as hematocrit, hemoglobin concentration and reticulocyte count, as well as the plasma iron and the total iron binding capacity, are shown in Table 2. The low binding capacity observed produced a high transferrin saturation (about 80%). The presence of reticulocytes in starling blood was almost constant.

In Table 3, the iron content in organs of specimens caught at three different times are shown and compared to values found also in our laboratory, in chickens and quails. In starlings, no sexual differences were noticed as, in winter, they are sexually inactive. So, the data was compared to non-laying females of other species, because of the lack of iron mobilization in this state. In the starling iron data there were significant difference ($P < 0.05$) in liver, spleen, intestines and feathers among the various groups. In general, when compared to chickens and quails, the differences were very significant ($P < 0.001$), starlings having 3 to 10 times more iron in their organs.

The whole iron content of the birds was analysed by evaluation of the metal in the ashes, obtained through incineration. The results can be seen in Table 4, where the iron values of female chickens and quails are also included. The high iron content in starlings was once more evident when the metal concentration, expressed for unit of body weight, was found to have over three times more iron than the other species studied.

Table 2. Hematological values and plasma iron data from starlings. The number of specimens analysed is indicated in parentheses. Mean ± SE. PI–plasma iron ; TIBC–total iron binding capacity; Ret–reticulocytes

Birds, date of capture	Body weight (g)	Hc (%)	Hb (g/100)	PI (μg Fe/100)	TIBC (μg Fe/100)	Ret. (%)
Group D	65.2 ± 1.1	45.2 ± 0.7	14.5 ± 0.3	137 ± 8	194 ± 12	4.0 ± 0.7
15 December 1981	(18)	(18)	(18)	(10)	(10)	(5)
Group C	66.7 ± 0.8	47.8 ± 0.6	16.2 ± 0.3	177 ± 13	187 ± 17	5.1 ± 0.5
4 November 1981	(19)	(19)	(21)	(12)	(11)	(9)

Table 3. Iron content in various organs of starlings, compared to values obtained from chickens and quails. Mean ± SE

Species No. specimens	μg Fe/g wet organ: Feathers	Liver	Pectoral muscle	Spleen	Bone marrow	Intestine	Heart
Starlings							
Group B, $N = 5$	563 ± 44	1672 ± 119	97 ± 11	273 ± 23	168 ± 18	46 ± 3	119 ± 7
Group C, $N = 6$	593 ± 45	1566 ± 230	106 ± 7	276 ± 17	193 ± 33	43 ± 3	112 ± 4
Group D, $N = 8$	387 ± 13	1311 ± 75	94 ± 8	348 ± 54	133 ± 13	36 ± 5	127 ± 9
Chickens (Shaver strain) Females, $N = 10$ (non-laying)	54 ± 4	178 ± 12	19 ± 6	154 ± 5	—	42 ± 4	56 ± 4
Quails Females, $N = 5$ (non-laying)	156 ± 19	170 ± 14	24 ± 2	104 ± 8	67 ± 2	29 ± 1	63 ± 6

Table 4. Iron content in the whole bird and for unit of body weight in different groups of starlings, when compared to non-laying female chickens and quails. Mean ± SE

Species No. specimens	Body weight	Iron content μg Fe/bird	μg Fe/g body wt
Starlings			
Group B, $N = 7$	58.1 ± 4.0	10,700 ± 1200	184 ± 19
Group C, $N = 5$	66.9 ± 1.5	10,485 ± 700	153 ± 10
Group D, $N = 5$	65.3 ± 1.3	12,060 ± 700	185 ± 10
Chickens (Shaver)			
Females, $N = 10$ (non-laying)	1296 ± 30.0	67,200 ± 4500	52 ± 4
Quails			
Females, $N = 4$ (non-laying)	140.7 ± 5.5	7230 ± 400	52 ± 2

DISCUSSION

A comparison of relative weights of organs, taken from different species, reflects the characteristic habits of a good flyer. So the pectoral muscle, heart size, liver and spleen of starlings were closer to values obtained by us from pigeons (Palomeque and Planas, 1978; Ramis and Planas, 1982), than to the values observed in chickens and quails (unpublished data, Palomeque and Planas, 1978).

The hematological values found were also to be similar to flapping flyers such as pigeons (Palomeque and Planas, 1978) and passeriforms (Palomeque *et al.*, 1980), than to sedentary domestic fowl and quails (Table 2). Reticulocytes were found in the starling blood, as is common in quail, pigeons, ducks and turkeys (Ramis and Planas, 1978), but not in chickens (Ramis and Planas, 1978; 1982), where there is a complete absence.

Plasma iron and total iron binding capacity, in the starlings, were similar to values found in males and non-laying females of other species (Planas, 1970)., No sexual differences were found, because the captures took place in winter, when the birds are too far from the breeding season. Plasma iron binding capacity was lower than the general range for other birds, where transferrin saturation is about 40–55% (Planas, 1970).

The high iron content in liver differed from other species of birds (Table 3) and mammals (Underwood, 1977). Our data fits in well with results obtained by Osborn (1979) in Great Britain, where he found seasonal changes, with a peak around September (1800 μg Fe/g liver) and the lowest point in April–June (600 μg Fe/g liver) during post breeding.

To the contrary, in India (Pilo, 1979) the start of migration for *Sturnus roseus* is also in April but the iron levels are at the lowest point. These iron levels are much lower (400–870 μg Fe/g wet liver) than those of the European starling. Osborn (1979) also suggested some type of relationship between iron stores, the breeding season (April–June) and moulting (June–September).

The iron content in feathers was 10 times higher than the values found in chickens and three times higher than those found in quails. The lowest values obtained in group D were observed in liver, bone marrow and intestines. Osborn (1979) does not give any values for iron concentration in feathers but he noted that liver stores increased after post-breeding, reaching a peak near the end of the moulting period (September).

Iron content in the pectoral muscle coincided with data from Pilo (1979), who observed a progressive increase during the pre-migratory state. The author attributed this to a rise in myoglobin. According to our data on *Sturnus vulgaris* (Palacios *et al.*, 1984), the myoglobin was significantly higher in recently arrived specimens (post-migratory state), due to their physical exercise which stimulates the synthesis of this protein (Lawrie, 1953).

The whole iron content per body weight of starlings (Table 4) was 153–185 ppm, which is 2–4 times higher than the values obtained from other species or from mammals (Underwood, 1977). We do not know if this exceptional richness in iron is a peculiarity of this species or if it is a general phenomenon in powerful flapping flyers. A similar study on pigeons could verify this second possibility.

In the present data, we did not find any significant variations in the tissue iron stores, which could have been attributed to the migratory process. However, in a yearly study, such as the one carried out by Osborn (1979), seasonal changes were evident in several essential metals in a permanent population, because of physiological events, such as reproduction or moulting. In our study this was not the case as the specimens were obtained only in fall or winter.

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A COMPREHENSIVE DESCRIPTION OF BRUSH BORDER MEMBRANE DEVELOPMENT APPLYING TO ENTEROCYTES TAKEN FROM A WIDE VARIETY OF MAMMALIAN SPECIES

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Abstract—1. Pieces of small intestine taken from rabbit, rat, mouse, guinea-pig, hamster and pig have been used to determine microvillus length in enterocytes located at different points along the crypt–villus axis. Thymidine labelling has also been used to convert measurements of enterocyte position into age of enterocyte.

2. Microvillus lengths showed lower and upper plateaux (a and $a + c$ respectively) with fairly rapid transition from one to the other defined by an exponential coefficient b. The mid-point of elongation (m) usually occurred within 60 μm of the crypt–villus junction.

3. Correlations were found to exist between a and m and between a, m and the depth of the intestinal crypt. Values of b and $bc/4$, the maximal rate of microvillus elongation, were also found to be correlated with the size of the crypt.

4. None of these parameters were related in any way to the villus height or enterocyte turnover time.

5. The possibility that some factor associated with the physical size of the crypt might be exerting positional and temporal control over the subsequent structural differentiation of enterocytes is discussed.

INTRODUCTION

Interest in the brush border membrane of the intestinal epithelial cell increased markedly once it became possible to study its fine structure using electron microscopy (Granger and Baker, 1950). Since then it has been possible, using electron microscopes with higher powers of resolution, to describe the internal architecture of individual microvilli both under near normal conditions (Bretscher and Weber, 1979) and following different types of disruptive treatment (Howe *et al.*, 1980; Mooseker *et al.*, 1980). From these studies has emerged a description of the microvillus as a complex structure consisting of about 20 actin filaments bound to each other and to the plasma membrane through a number of proteins some of which change their characteristics in the presence of Ca^{2+} (see Bretscher, 1983 for a recent review).

Microvilli also increase in length as enterocytes migrate from their point of origin in the intestinal crypt to their point of extrusion from the villus tip (Brown, 1962; Trier, 1967), presumably as actin monomers add to the end of core filaments attached to the plasma membrane at the microvillus tip (Mooseker and Tilney, 1975; Mooseker *et al.*, 1982). During this time the microvillus content of membrane hydrolases increases (Dahlqvist and Nordström, 1966; Webster and Harrison, 1969; Weiser, 1973) and the microvillus membrane later becomes able to transport nutrients (Kinter and Wilson, 1965; King *et al.*, 1981). The time courses with which these latter events take place are already known but there is no comparable work describing the kinetics of microvillus elongation. The main object of the present work was to provide this information using intestines chosen from a number of species to provide a variety of intestinal architecture and enterocyte turnover time.

In this work it was found possible to fit logistic growth curves to data describing the complete process of microvillus elongation. Predictive statements could then be made concerning the relation between constants used to describe these curves and the physical size of the intestinal crypts. Preliminary accounts of some of these results have already been published in abstract form (King *et al.*, 1983a; Smith *et al.*, 1983).

MATERIALS AND METHODS

Animals

AJAX/IAP mice (35 g) (*Mus musculus*) and guinea-pigs (300 g) (*Cavia parcellus*) were bred at Babraham from stock originally purchased from G. D. Searle, High Wycombe, Bucks., and Redfern Animal Breeders, Frant, Tunbridge Wells, Kent. Hamsters (75 g) (*Mesocricetus auratus*) were bought from Wrights of Essex, Latchingdon, Chelmsford, Essex and rabbits (2.5 kg) (*Oryctolagus cuniculus*) came from Morton Commercial Rabbits, Stansted, Essex.

Rats (*Ratus nowegicus*) of the Sheffield strain were weaned at 21 days onto isoenergetic diets containing 200 or 50 g protein kg^{-1} for a period of four weeks before use. At the time of experiment rats maintained on a high protein diet (H) weighed 150 g while those maintained on a low protein diet (L) weighed 45 g. The composition of the diets used is given in the paper by Syme (1982).

Pigs born at Babraham of the large white strain, taken from the sow on day 14, were given excess food to eat for a period of three days before being placed on either a high (H) or low (L) energy containing diet (H/L = 2). Changes in environmental temperature were started on day 17, final temperatures of 35 or 10°C being reached by day 28. The

four groups of pigs used for experiment at 7 weeks of age consisted of those adapted to H and L diets at temperatures of 35 or 10°C (35H, 35L, 10H and 10L). The average weights of these animals at the time of the experiment were 10.8, 7.6, 8.3 and 5.6 kg respectively. Further details of the procedure used to adapt these animals to changes in diet and temperature are given in the paper by Smith *et al.* (1982).

Experimental

Mice, hamsters and guinea-pigs were killed by a blow on the head, rats were anaesthetized with ether and rabbits were killed by the intravenous injection of pentobarbitone sodium. Pigs were pre-medicated with Ketamine before being killed by the intracardiac injection of sodium barbiturate. All animals were fasted but allowed free access to water for 24 hr before use. Pieces of mid-intestine of mice, hamsters, guinea-pigs and pigs were taken for analysis. Pieces of mid-jejunal tissue from rats and distal ileum from rabbits were taken to allow comparison with work published previously (Syme, 1982; King *et al.*, 1981).

Electron microscopical determination of microvillus length

Pieces of small intestine were fixed in phosphate buffer containing 4% v/v glutaraldehyde and 2% w/v sucrose within 5 min of killing the animal. Strips of these tissues were post-fixed in Veronal buffer containing 1% w/v osmium tetroxide for a period of 60 min and the post-fixed tissue dehydrated, embedded in Araldite and sectioned for examination in a Philips 400 electron microscope. Only those areas of tissue showing perpendicularly sectioned microvilli were used for measurement. Individual positions on the crypt–villus axis chosen for measurement of microvillus length were determined by recording the number of calibrated steps taken to move from the base of the crypt to the tip of the villus. Three to four crypt–villus units were analysed from tissue taken from each animal.

Thymidine labelling experiments

Estimates of enterocyte lifespan were obtained by the intraperitoneal injection of 1 μCi g^{-1} tritiated thymidine ([methyl-^{3}H]thymidine 40–60 Ci mmole^{-1}; Amersham International plc, Amersham, Bucks) into animals killed at known times afterwards. Sections of fixed material were processed for autoradiography and the position of the leading edge of migrating thymidine labelled enterocytes, measured using a curtain eyepiece micrometer attached to a light microscope, related to the time following thymidine injection.

Parallel estimates of crypt depth and villus height were made on serial sections of intestine stained with haematoxylin and eosin. Mean values obtained from these measurements were used to calculate enterocyte lifespan assuming migration rate to remain constant throughout.

Fitting logistic growth curves to experimental data

Logistic curves having the form $y = a + c/\{1 + \exp[-b(x - m)]\}$, where a is the lower asymptote, $a + c$ is the upper asymptote, m is the point of inflexion and b is the exponential coefficient, were fitted to experimental measurements of microvillus length, made along the crypt–villus axis, using the maximum likelihood program MLP (c. 1982 Lawes Agricultural Trust, Rothamsted Experimental Station).

In this analysis it was possible to fit common non-linear parameters b and m within species but there was considerable between-animal variation for the linear parameters a and c. Results summarized in the figures represent common curves fitted to data pooled for each species. Rankit plots of crypt depth, villus height, enterocyte lifespan and the four constants describing logistic growth curves on both time and distance bases showed these variables to be normally distributed. Correlations between the constants b, m, a and c and measurements of crypt depth, villus height and enterocyte lifespan were calculated using standard techniques of analysis.

RESULTS

Intestinal structure and enterocyte lifespan

The present work uses a mixture of species and environmental circumstance to produce a total of 10 different experimental conditions for the measurement of brush border membrane development. Initial measurements of intestinal architecture and enterocyte lifespan obtained from these animals are summarized in Table 1.

There was, between the six species, a 2- to 4-fold variation in crypt depth, villus height and enterocyte lifespan. Changing the energy intake from L to H within a species caused a 15–25% increase in crypt depth, a 15–60% increase in villus height and no significant change in the time taken for enterocytes to migrate to the tips of villi. Changing the environmental temperature of pigs from 10 to 35°C caused a 30–40% fall in enterocyte lifespan without marked effect on crypt depth or villus height. There appeared from these results to be no obvious relation between the three measured variables. This was verified by calculating correlation coefficients for the three different variables.

Microvillus elongation during development

Pieces of tissue taken from the same animals as those used to provide structural data summarized in Table 1 were also processed for electron microscopy. Measurements of microvillus length made at different points along the crypt–villus axis were then pooled and the results plotted in Fig. 1.

Features of microvillus development common to all species included an initial period during which

Table 1. Intra and interspecies variability in intestinal structure and enterocyte lifespan

Species	Crypt depth (μm)	Villus height (μm)	Enterocyte lifespan (hr)
Rabbit	105	510	96
Rat H	121	450	56
L	105	300	56
Mouse	80	346	64
Hamster	168	602	40
Guinea-pig	179	347	81
Pig 10H	276	539	66
10L	227	339	78
35H	302	422	46
35L	243	371	45
Correlation matrix			
Crypt depth	1.000	0.149	−0.285
Villus height		1.000	−0.123
Enterocyte lifespan			1.000

Pieces of small intestine taken from animals either before or after the intraperitoneal injection of 1 μCi g^{-1} tritiated thymidine were processed for determination of enterocyte lifespan and crypt–villus structure as described in the text. Measurements of crypt depth and villus height give the means of 65–145 determinations on tissue taken from 11–15 animals. Values of enterocyte lifespan are generally based on work carried out on 7–12 animals. The one exception was with pigs, where the size prevented more than two animals being used in each group for determination of enterocyte lifespan. The subsidiary codings for rats (H and L) and pigs (10H, 10L, 35H and 35L) refer to changed environmental circumstances described in the text.

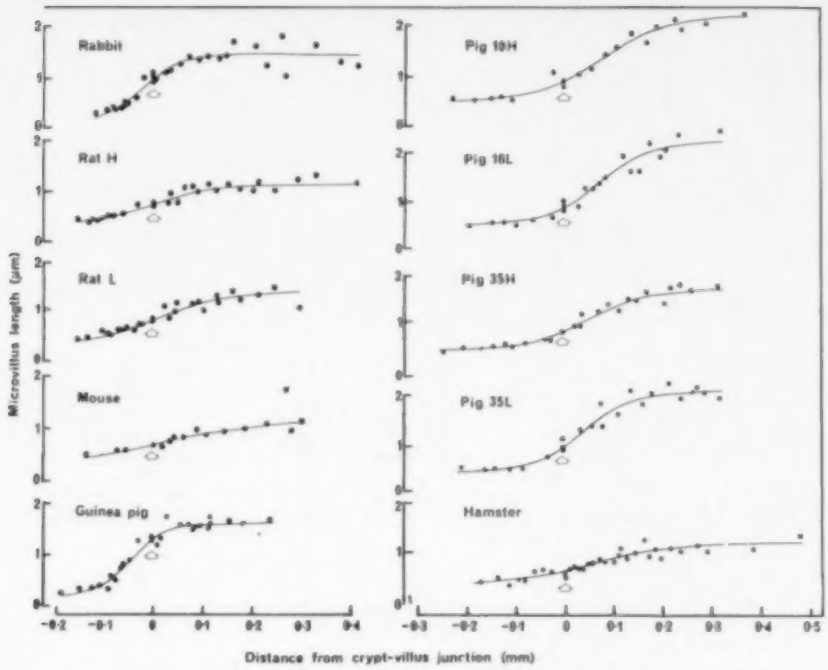

Fig. 1. Microvillus elongation in migrating enterocytes. Pieces of intestine taken from six different species were fixed and prepared for EM examination as described in the text. Microvillus length was then determined at different points along the crypt–villus axis. Each point gives the mean of five measurements from 3–8 animals pooled in order of increasing microvillus length. Common logistic growth curves have been fitted to each set of pooled data. The arrows show the position of the crypt–villus junction. H and L: differences in diet described in the text. 35 and 10: environmental temperatures at which pigs were kept.

elongation was minimal, an intermediate period during which elongation was rapid and a final period, when enterocytes had reached the upper part of the villus, when microvillus elongation again became negligible. The length of microvilli detected in enterocytes encountered near the base of crypts was, however, always in the region of 0.3–0.5 μm. It was concluded from this that the initial process leading to the formation of immature microvilli must take place rapidly.

The maximum microvillus length reached during natural development appeared to vary between species. Fully formed microvilli from rats, hamsters and mice were usually of the order of 1 μm; those from guinea-pigs and rabbits reached 2 μm while those from pigs often exceeded 2.5 μm. Further quantitative assessment of these supposed differences was made by fitting logistic growth curves to individual sets of experimental data. Constants describing the characteristics of these curves are given in Table 2.

Values for a, the predicted minimal size of an enterocyte microvillus, were in all cases positive, the mean value for the 10 different conditions being 0.34 ± 0.05 μm. This verified what had been suspected previously, that the initial appearance of microvilli on the surface of enterocytes takes place rapidly. Values for b and c, both of which measure different aspects of microvillus growth following initial formation, showed a 2- to 3-fold variation between species. Values for m, the point at which microvillus elongation is taking place at maximal rate, showed little variation when considered in relation to the total length of the crypt–villus unit (400–800 μm). Maximal rates of change in microvillus structure usually occurred in enterocytes during migration over the basal part of the villus. The rather large negative value for m calculated for the guinea-pig is considered to be somewhat anomalous in this respect. It is extremely difficult to specify the exact position of the crypt–villus junction in this species and this could have introduced error into the estimations.

The correlation matrix describing the interactions that exist between the four parameters a, c, b and m is also shown in Table 2. The only parameters to show significant correlation in this analysis are a and m ($P < 0.0005$). The initial burst in microvillus assembly appears in this case to reflect the long-term potential of the cell to extend individual microvilli.

Subsequent analysis was carried out to test which, if any, of the logistic curve functions might be related to earlier measurements of crypt depth, villus height and enterocyte lifespan. The results obtained from this analysis are summarized in Table 3. None of the calculated parameters were in any way related to the physical size of the villus or to the time taken for enterocytes to migrate to the villus tips. There were, however, statistically significant correlations between a and m and the size of the intestinal crypt ($P < 0.01$ and < 0.05 respectively).

Table 2. Logistic growth curve constants describing microvillus elongation

Species	a (μm)	c (μm)	m (μm)	b (μm^{-1})
Rabbit	0.13	1.33	−19.1	0.0267
Rat H	0.37	0.71	12.0	0.0187
L	0.24	1.16	7.5	0.0139
Mouse	0.15	1.09	−2.5	0.0084
Hamster	0.37	0.88	61.9	0.0118
Guinea-pig	0.17	1.47	−44.8	0.0290
Pig 10H	0.51	1.72	78.1	0.0151
10L	0.54	1.69	73.7	0.0185
35H	0.46	1.25	52.9	0.0169
35L	0.45	1.60	34.7	0.0196
Correlation matrix				
a	1.000	0.3476	0.9052*	−0.2252
c		1.000	0.2485	0.3331
m			1.0000	−0.5042
b				1.0000

Logistic curves having the form $y = a + c/\{1 + \exp[-b(x - m)]\}$ were fitted to data described in Fig. 1. a, the predicted minimal length of an enterocyte microvillus. c, the maximal change occurring in microvillus length once it has formed. m, the position on the crypt–villus axis where microvillus elongation is taking place at a maximal rate, distance being measured in relation to the crypt–villus junction. b, the exponential coefficient describing the positional dependency of microvillus elongation. Only one of the correlation coefficients was statistically significant ($P < 0.0005$*).

Kinetic aspects of microvillus development

Previous comparisons between the various parameters used to describe microvillus elongation have been related to enterocyte position on the crypt–villus axis. These measurements of enterocyte position can be converted to estimates of enterocyte age by calculating the time taken for enterocytes to migrate from base of crypt to tip of villus. This was done in the present experiments to test whether distance dependent correlations, seen previously between a, m and crypt depth, would also apply when related to age of the enterocyte. The results of carrying out these transformations on the general appearance of the logistic growth curves is shown in Figs 2 and 3.

The fit between the logistic growth curves and the experimental data is in all cases good. Presenting results on a time base can, however, be seen to cause marked changes in the way different sets of data relate to each other. This is best seen by comparing the time taken for enterocytes to migrate to their respective crypt–villus junctions. Such times range from about 9 hr in the hamster to over 31 hr in pigs maintained on a low energy intake at an environmental temperature of 10°C. A summary of the logistic curve functions calculated to fit these different sets of experimental results is given in Table 4.

The logistic curve constants a and c, which remain unchanged when calculated on a time base, have been included in the present table for the sake of completeness. The values of m and b, calculated on a time base, both show a 3- to 4-fold variation between species. This range of variation is greater for b than

Table 3. Relating logistic curve functions to physical measurements of intestinal structure

Logistic curves function	Correlation coefficient: Crypt depth	Villus height	Enterocyte lifespan
a	0.8019†	0.1776	−0.4358
c	0.5870	−0.2209	0.3863
m (μm)	0.6642*	0.3536	−0.4802
b (μm^{-1})	0.0788	−0.0682	0.6206

Data taken from Tables 1 and 2 were used to construct a correlation matrix from which were extracted the correlation coefficients shown above. Two of these coefficients were found to be statistically significant ($P < 0.05$* and $P < 0.01$†).

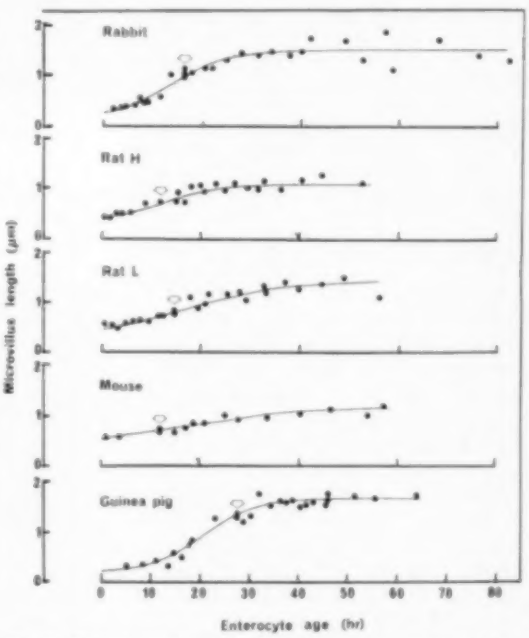

Fig. 2. Time dependency of microvillus elongation in rabbit, rat, mouse and guinea-pig small intestine. Tritiated thymidine (1 μCi g^{-1}) was injected intraperitoneally into animals in order to label enterocytes entering their S-phase prior to mitosis. The positions of these labelled cells were then followed to determine the time taken to migrate to the villus tips. Further details of the methods used to calculate enterocyte lifespan are given in the text. Results obtained enabled enterocyte position on the crypt–villus axis to be converted into estimates of enterocyte age. Common curves have then been fitted to each set of transformed data. Arrows show the times taken for enterocytes to reach their respective crypt–villus junctions.

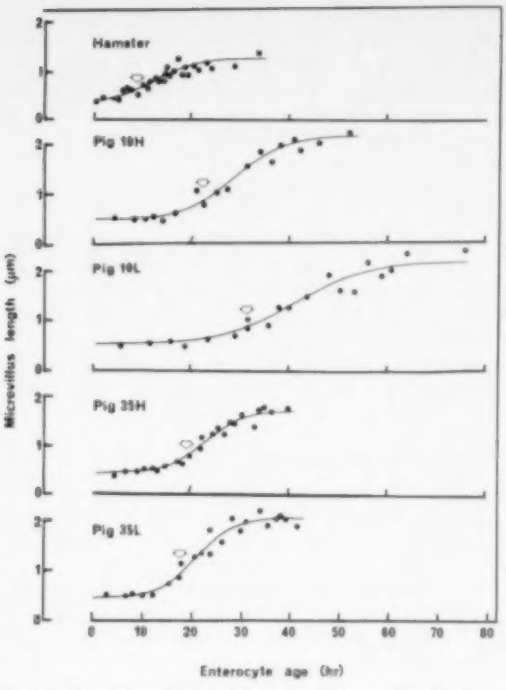

Fig. 3. Time dependency of microvillus elongation in hamster and pig small intestine. The method used to calculate the time dependency of microvillus elongation, the fitting of logistic growth curves to transformed data and the explanation of the signs and symbols used were all as described in the legend to Fig. 2.

when calculated using distance measurements. The values for m cannot be compared in this way, however, since they measure time taken to migrate from the base of crypts while distance measurements were made from the crypt–villus junction.

The correlation matrix describing interactions existing between the four calculated parameters show significant correlation between a and m ($P < 0.05$) and c and m ($P < 0.02$). The correlation between a, the initial formation of the immature microvillus, and m, the time taken to express the maximum growth rate, was not however as significant as that seen when measured on a distance basis (Table 2). These results suggest that the maximum ability of the enterocyte to extend its microvillus membrane is primarily related to a by distance, but that time determines the relation betwen m and c, the maximum amount of microvillus elongation seen to take place.

Further analysis was carried out to test for correlation between measurements of crypt depth, villus height, enterocyte lifespan and the logistic curve constants m and b determined on a time base. The results of this analysis are shown in Table 5. Neither of the logistic curve functions showed any correlation with villus height or enterocyte lifespan. This confirms previous results where m and b had been calculated in relation to distance measured from the crypt–villus junction (Table 3). The correlation found between m and crypt depth was also in agreement with that calculated previously. There was, in addition, a significant correlation ($P < 0.05$) between crypt depth and b, the exponential coefficient used to describe the rapidity with which microvillus growth

Table 4. Logistic growth curve constants describing the time dependency of microvillus elongation

Species	a (μm)	c (μm)	m (hr)	b (hr^{-1})
Rabbit	0.13	1.33	13.4	0.1712
Rat H	0.37	0.71	13.1	0.1831
L	0.24	1.16	15.9	0.0988
Mouse	0.15	1.09	11.1	0.0561
Hamster	0.37	0.88	11.9	0.2247
Guinea-pig	0.17	1.47	20.7	0.1886
Pig 10H	0.51	1.72	28.7	0.1865
10L	0.54	1.69	41.4	0.1351
35H	0.46	1.25	22.6	0.2646
35L	0.45	1.60	20.8	0.2673
Correlation matrix				
a	1.0000	0.3476	0.6925*	0.4821
c		1.0000	0.7635†	0.1210
m			1.0000	0.0863
b				1.0000

The linear parameters a and c have the same values as described previously in Table 2. The non-linear parameters m and b have been recalculated according to age of enterocyte, this being determined as described in the text. Two of the determined correlation coefficients were statistically significant ($P < 0.05$* and $P < 0.02$†).

Table 5. Relating logistic curve functions determined on a time basis to physical measurements of intestinal structure

Logistic curves function	Correlation coefficients: Crypt depth	Villus height	Enterocyte lifespan
m (hr)	0.6644*	−0.2234	0.2240
b (hr^{-1})	0.6899*	−0.4291	−0.4058

Data from Tables 1 and 4 were used to construct a correlation matrix. Correlation coefficients for the linear parameters a and c were as shown in Table 3. Correlation coefficients for both m and b against crypt depth were statistically significant ($P < 0.05$*).

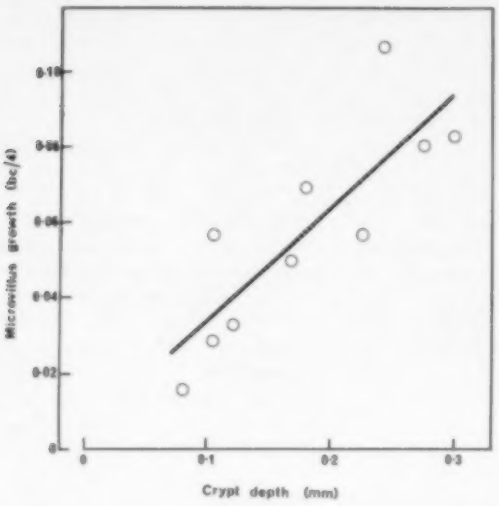

Fig. 4. Crypt depth dependency of microvillus elongation. Values for the maximum rate of microvillus elongation ($bc/4\ \mu m\ hr^{-1}$) are plotted against crypt depth for the 10 different experimental conditions used. The straight line fitted to these points has a correlation coefficient of 0.83 ($P < 0.005$).

takes place. This latter correlation extends significantly the control intestinal crypts appear to exert over the subsequent behaviour of migrating enterocytes.

Maximal rates of microvillus growth

Previous work shows several of the parameters used to describe microvillus elongation to be correlated with the size of crypts from which enterocytes originate. In this case it is interesting to determine whether the size of crypts also correlates with the maximal rates at which microvilli elongate ($bc/4$). This derived parameter is plotted against measurements of crypt depth in Fig. 4.

The results obtained show crypt depth and the maximal rate of microvillus elongation to be directly related with a correlation coefficient of 0.8295 ($P < 0.005$). Regression analysis of this data produces a straight line having a slope of 0.295 ± 0.070 and an intercept which is not significantly different from zero ($0.005 \pm 0.014\ \mu m\ hr^{-1}$).

DISCUSSION

The maximal rate of microvillus elongation (m)

Microvillus elongation and the appearance of both aminopeptidase *N* and isomaltase activities in rat enterocyte brush border membranes have been shown previously to reach maximal rates of expression shortly after passing onto the basal regions of the villus (King *et al.*, 1983b). Positional measurements of *m* in that instance were found not to correlate with a 15% difference in crypt depth associated with change in diet. Nevertheless this can be demonstrated using intestines taken from different species where crypt depth varies by a factor of four (Table 1). The significant correlation seen between crypt depth and time based estimates of *m* is less easy to interpret since *m* in this case includes time spent by enterocytes within the crypt and this could itself be related to crypt depth. Further analysis of this possible secondary relationship, though feasible, was not attempted in the present work.

All the present results have been obtained using tissue taken from physiologically normal animals. Separate studies suggest, however, that the position of *m* on the crypt–villus axis might shift towards the crypt under conditions where crypt depth increases in response to injury (M. W. Smith and B. Miller, unpublished results). Whether or not this inverse correlation represents adaptation or submission to the effects of dietary insult has yet to be established.

The predicted minimal length of enterocyte microvilli (a)

The lower asymptote *a* of the logistic function is not necessarily equal to the smallest microvillus length measured at the base of the crypt. Nevertheless, it was predicted that *a* might be equal to zero, that this would exclude any correlation between *a* and the other variables and that microvillus growth would begin at a point where mitosis stopped. All of these predictions proved wrong, estimates of *a* being always positive and highly correlated with estimates of *m* (Table 2). The additional correlation found between *a* and crypt depth was also highly significant (Table 3).

The unexpected finding of positive values for *a* can best be explained by suggesting that the initial formation of microvilli takes place rapidly. This could occur from the sudden polymerization of pre-formed actin in a way analogous to that reported to take place *in vitro* (Mooseker *et al.*, 1982).

But why should this burst in microvillus formation, taking place at a rate at least ten times than seen for 'normal' elongation, be correlated to crypt depth and to calculated values for *m*? One explanation would be to suggest that both the initial appearance (*a*) and the mid-point of elongation (*m*) of enterocyte microvilli reflect different aspects of the same potential to differentiate imposed on cells at or near the time of birth. The capacity of the surrounding environment to impose such a potential would, by this hypothesis, be directly dependent upon the quantity of crypt tissue present.

Exponential coefficient describing microvillus elongation (b)

Values of *b*, which are inversely related to the time taken to change microvillus length from *a* to $a + c$, are significantly correlated with crypt depth (Table 5). It is suggested from this result that the postulated differentiation potential of the crypt specifies the duration of the differentiation response (*b*) as well as the point on the crypt–villus axis where that response is expressed at maximal rate (*m*).

Decreasing the time during which enterocytes differentiate can have adaptational advantages, particularly when crypt cell production rate is being stimulated unduly. Enterocytes produced under these conditions will migrate quickly to areas on the villus

where the potential to differentiate is no longer effective. Increasing b as an adaptational response to this situation will allow such cells to exploit their limited time within the differentiation zone more efficiently.

Multiplying the elongation coefficient (b) by the maximal amount of microvillus elongation calculated to take place after its initial formation (c) allows one to estimate the maximal rate of microvillus extension ($bc/4$). This product is directly proportional to the depth of the crypt from which the enterocyte migrated ($P < 0.005$; Fig. 4).

Possible factors controlling the structural differentiation of enterocytes

The impressive amount of data showing biosynthetic properties of mature enterocytes to differ from those found in proliferating cells and the large amount of information available describing how crypt cell proliferation rate can be changed has not, as yet, led to any significant advance in our understanding of how enterocyte differentiation takes place *in vivo* (Lipkin, 1981). The present approach to this problem was to make a quantitative assessment of how enterocytes undergo structural differentiation under so-called normal conditions. The results produced highlight the importance of crypt size in determining the overall pattern of development, but they give no information about how this message might be transmitted. All that can be offered by way of explanation at the moment is one negative statement and one tentative suggestion.

The negative statement concerns the proliferative activity of the crypt which has, in the past, often been thought to be directly related to the depth of crypt. Present work shows increase in crypt depth to be correlated with m by position mainly and with b and $bc/4$ measured on a time base, but these correlations cannot be related to the proliferative activity of the crypt. This is seen most clearly for pigs, animals in the cold maintained on a low energy intake having deep crypts and small villi and yet one of the slowest enterocyte turnover times measured. A similar independence between cell proliferation rate and the ability to transfer positional information has also been reported for limb development in the chick (Smith *et al.*, 1978).

An alternative source for information control over enterocyte differentiation lies in mesenchymal tissue underlying crypt enterocytes. Depleting fetal intestine of mesenchyme leads to impaired development of the epithelium (David, 1972) and neoplastic colonic lesions are associated with known changes in pericryptal fibroblasts (Tutton, 1973; Kaye *et al.*, 1971; Lane *et al.*, 1971). The number and properties of fibroblasts might also depend on crypt depth and this could indirectly give rise to the types of correlation noted above. The design of experiments to test this possibility might now seem warranted.

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THE EFFECT OF FOOD DEPRIVATION ON SEASONAL CHANGES IN THE METABOLIC RATE OF THE LIMPET, *CELLANA TRAMOSERICA*

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Abstract—1. The metabolic rates of the intertidal limpet *Cellana tramoserica* were compared when limpets were caged at high densities in the field and when they were left uncaged at natural densities.

2. Food deprivation caused by caging had different effects on metabolism in different seasons.

3. During winter, severe food deprivation caused somatic growth to cease and eliminated the "elevated" metabolism (i.e. "temperature acclimatization") usually found in this season.

4. During spring, caging did not cause a reduction in metabolism apparently because the rate of gonad synthesis did not decrease when limpets were deprived of food.

5. These results provide further support for a metabolic model which suggests that seasonal changes in oxygen consumption are due to changes in the rate of biosynthesis rather than to "temperature acclimation".

INTRODUCTION

Many authors (e.g. Newell, 1970; Vernberg and Vernberg, 1972; Prosser, 1973; Precht *et al.*, 1973) have suggested that natural selection has favoured the evolution of a relatively temperature-independent "optimal" metabolic rate in many ectotherms. However, this view has been widely criticized (Dunbar, 1968; Holeton, 1974; White, 1975; Everson, 1977; Parry, 1978, 1983; Clarke, 1979, 1980; Torres *et al.*, 1979) as it implies that, at low temperatures, metabolic rates must be increased at the expense of other energy budget components, such as growth, for no obvious benefit. Indeed, natural selection seems more likely to favour maximization of growth than the wasteful maintenance of a high metabolic rate (Newell and Branch, 1980). However, as I have shown elsewhere (Parry, 1983), these conflicting views can be reconciled. A high growth rate necessarily causes metabolism to increase significantly above the maintenance level, as biosynthesis is usually only about 70–80% efficient. Thus where increased metabolic rates are observed at low temperatures this may be a direct consequence of enhanced growth at the lower temperature (see Parry, 1983 for details).

Empirical support for the view that the maintenance of a temperature-independent metabolic rate is adaptive has derived from two sources. First, from studies of "temperature (thermal) acclimation" or "temperature (seasonal) acclimatization", in which an ectotherm adjusts its metabolic rate in a (usually) compensatory manner following exposure for several days or weeks to a changed temperature. Secondly, from the reportedly high metabolic rates of ectotherms which occur in cold climates.

Most criticisms of the concept of the adaptive value of optimum metabolic rates have come from workers studying the metabolic rates of latitudinally separated ectotherms (reviewed by Clarke, 1980). These workers have demonstrated that most Arctic and Antarctic ectotherms do not have metabolic rates which are elevated compared to temperate or tropical species. Early work which showed elevated metabolism in Antarctic fishes has been largely discredited (Holeton, 1974; also see Parry, 1983). However, despite the difficulties outlined above, the concept of an optimum metabolic rate is still frequently used to explain seasonal changes in ectotherm metabolism. The present paper elaborates an alternative hypothesis to explain seasonal changes in the metabolism of ectotherms. This hypothesis (Parry, 1978) suggests that seasonal changes in ectotherm metabolism result from changes in growth rate rather than from acclimation. "Acclimation" of metabolic rate is then re-interpreted as being the result of temperature-mediated changes in growth rate, rather than as a direct adaptation of metabolic rate to temperature (Parry, 1983).

This paper provides a partial test of a previous model of seasonal changes in the metabolism of the intertidal limpet *Cellana tramoserica* (Parry, 1978). This model accurately predicts seasonal changes in the metabolism of this limpet using the following assumptions:

(1) Maintenance metabolism at a particular temperature does not change seasonally and can be estimated from the metabolic rate during a period in which no growth occurs.

(2) The efficiency of biosynthesis is 67%, i.e. the synthesis of 2 J of tissue creates 1 J of heat.

If the above model is correct then seasonal changes in metabolic rate will not occur if seasonal changes in growth rate are prevented. In this study seasonal changes in the metabolism of limpets which show their normal seasonal pattern of growth are compared with those in which growth is minimized by caging limpets in the field at high densities.

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MATERIALS AND METHODS

All limpets were collected from a rocky shore adjacent to Griffith Point near San Remo, 80 km south east of Melbourne, Australia.

Limpets (shell length 3.2 ± 0.2 cm) were placed in cages which measured $40 \times 40 \times 4$ cm (see Parry, 1982a for details). Limpets were caged at densities of 5 limpets/cage (31 limpets/m^2), 40 limpets/cage (250 limpets/m^2) and 80 limpets/cage (500 limpets/m^2) from 29 April 1979 till 20 June 1979 (52 days); 10 and 40 limpets/cage from 29 July 1979 till 24 October 1979 (87 days); 10 and 40 limpets/cage from 21 June 1980 till 29 August 1980 (69 days); 80 limpets/cage from 27 May 1982 till 24 August 1982 (89 days), and 80 limpets/cage from 27 May 1982 till 24 October 1982 (150 days).

Aerial oxygen consumption of limpets was measured at 15 ± 0.1°C using constant pressure respirometers (Parry, 1978). During June 1979, October 1979, August 1980 and August 1982 limpets were collected on a falling tide from within cages and from areas adjacent to cages. These limpets were placed individually on plastic discs and returned to the laboratory within 30 min. Measurements of aerial oxygen consumption were completed during the period of the low tide in which limpets were collected. Limpets which altered their positions during an experiment were not included in subsequent analysis. Following measurements of oxygen consumption limpets were kept for 3 days in aerated seawater to ensure that their gut contents were completely voided, and then their shell lengths, dry tissue weights and ash contents were determined as described by Parry (1978).

Gonads were dissected from limpets collected in August 1980, August 1982 and October 1982 and the dry weights of gonads and somatic tissues determined separately. *C. tramoserica* reaches sexual maturity at a shell length of 2.36 cm (Parry, 1978), consequently regressions of gonad weight (G) against shell length (L) of the form $G = b(L - 2.36)$ were calculated for limpets collected in August 1980, August 1982 and October 1982. Indices of reproductive condition were obtained by using these regressions to calculate the gonad weight of a 3.25 cm limpet.

Rates of metabolic heat loss were calculated from oxygen consumption by assuming an energy equivalent of 20.2 J/ml O_2 (Elliott and Davison, 1975).

RESULTS

Covariance analysis of regressions of log (oxygen consumption) vs log (dry tissue mass) revealed no significant differences between limpets caged at different densities on the following dates: June 1979, October 1979, and August 1980 (Fig. 1). Similarly, in June 1979 and October 1979 covariance analysis of regressions of log (dry tissue mass) vs log (shell length) revealed no significant differences between limpets caged at different densities (Table 1). Furthermore, in June and October 1979 ash contents of limpets caged at different densities showed no significant differences (ANOVA, $P > 0.05$, Table 1). The similarity of oxygen consumptions, dry tissue

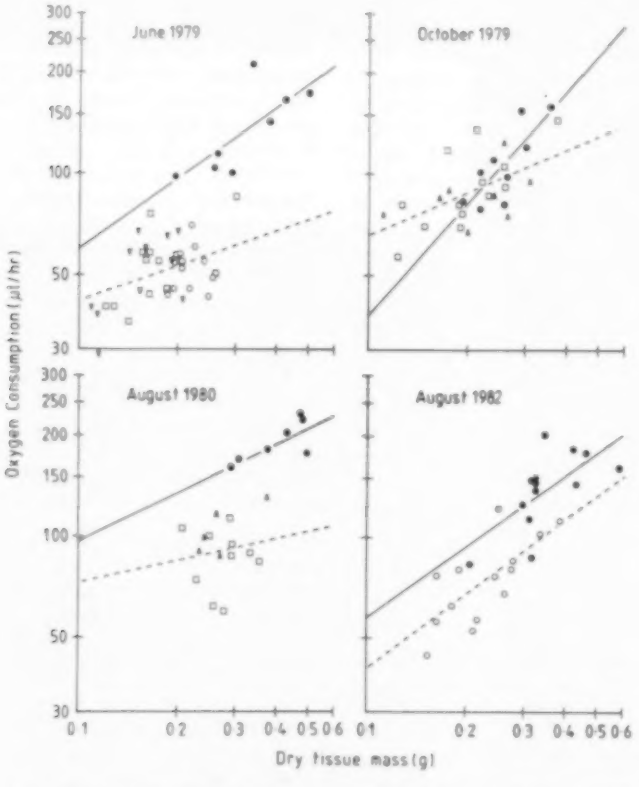

Fig. 1. Regression equations of log (oxygen consumption) against log (dry tissue mass) for caged (dashed line) and uncaged (solid line) limpets. ●, Uncaged limpets; ▽, 5 limpets/cage; △, 10 limpets/cage; □, 40 limpets/cage; ○, 80 limpets/cage.

Table 1. Dry tissue masses of 3.25 cm limpets [calculated from log (dry tissue mass) vs log (shell length) regressions of N limpets] and ash contents of caged and uncaged limpets

Date	Limpets/cage	N	Dry tissue mass (g) of 3.25 cm limpet ± 95% C.L.	Ash Content ± 95% C.L.
June 1979	5	13	0.180 (0.154–0.208)	36.5 ± 0.37
	40	17	0.198 (0.182–0.215)	36.9 ± 0.37
	80	17	0.197 (0.184–0.210)	36.9 ± 1.13
	uncaged	17	0.274 (0.255–0.295)	30.3 ± 0.18
October 1979	10	9	0.209 (0.148–0.296)	30.6 ± 3.68
	40	19	0.237 (0.197–0.284)	29.9 ± 0.54
	uncaged	16	0.309 (0.257–0.371)	19.0 ± 0.98
August 1980	10	18	0.268 (0.249–0.288)	25.3 ± 0.80
	40	20	0.241 (0.226–0.260)	27.0 ± 0.35
	uncaged	18	0.360 (0.332–0.390)	16.0 ± 0.27
August 1982	80	21	0.178 (0.161–0.196)	24.0 ± 0.31
	uncaged	21	0.309 (0.291–0.328)	15.6 ± 0.15

masses, and ash contents at all cage densities in June 1979 and again in October 1979, suggests that the amount of food available to limpets is not greatly affected by the density at which limpets are caged. This unusual result is probably caused by complex interactions between limpets and algae (see Parry, 1982a, for detailed discussion), and has led to a pooling of all results for caged limpets obtained in June 1979 and, similarly, for October 1979.

Although the oxygen consumption of limpets caged at different densities in August 1980 did not differ significantly, those caged at a density of 10/cage had a significantly higher dry tissue weight and a significantly lower ash content than those caged at a density of 40/cage (Table 1). Consequently the metabolic rates of limpets caged at densities at 10/cage and 40/cage in August 1980 have been considered separately (Fig. 2(A)).

Covariance analysis revealed significant differences between regressions of log (oxygen consumption) vs log (dry tissue mass) of caged and uncaged limpets in June 1979, August 1980, August 1982, but not in October 1979 (Fig. 1). Similar analysis of regressions of log (dry tissue mass) vs log (shell length) indicated that caged limpets always had lower dry tissue masses than uncaged limpets (Table 1). The poorer "condition" of caged compared to uncaged limpets was also indicated by the significantly higher ash contents of caged limpets during all seasons (ANOVA, $P < 0.05$, Table 1).

Covariance analysis of regressions of log (oxygen consumption) vs log (dry tissue mass) revealed significant differences between August 1980 and August 1982, for uncaged limpets (Figs 1 and 2). Covariance analysis of regressions of log (dry tissue weight) vs log (shell length) also revealed significant differences between August 1980 and August 1982 for uncaged limpets (Table 1).

DISCUSSION

The concepts of temperature acclimation and temperature acclimatisation should be applied only to changes in standard or "maintenance" metabolic rates (Block and Young, 1978). In the present study, only standard metabolic rates are considered as limpets which were active during measurements of oxygen consumption were excluded from the analysis.

Table 2. Indices of reproductive condition

Date	Limpets/cage	Sample size N	Gonad mass (g) of a 3.25 cm limpet	% of limpets with gonad >0.005 g
June 1979	5	13	0.0	0
	40	17	0.0	0
	80	17	0.0	0
	uncaged	17	0.0	0
October 1979		No Data		
August 1980	10	18	0.007	45
	40	20	0.003	20
	uncaged	18	0.031	100
August 1982	80	21	0.002	5
	uncaged	21	0.006	48
October 1982	80	20	0.005	40
	uncaged	20	0.045	100

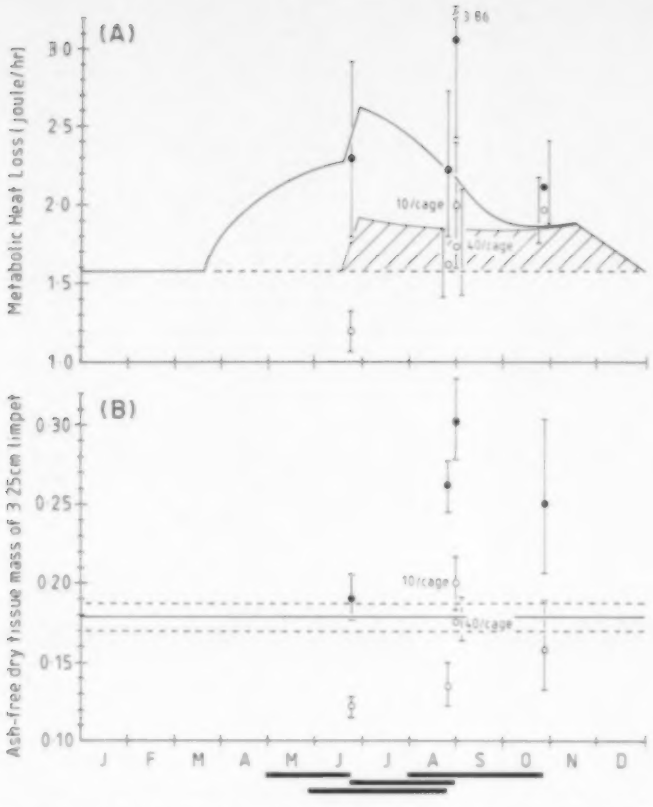

Fig. 2. (A) The predicted and observed seasonal variation in metabolic rate of a 0.25 g limpet. Predicted seasonal changes are from Parry (1978). The dashed line estimates the maintenance metabolic rate, and the hatched region estimates the metabolic heat loss due to gonad synthesis. Observed metabolic rates of uncaged (●) and caged (○) limpets are estimated from regressions in Fig. 1. Error bars are 95% confidence limits. Periods limpets were caged prior to measurements of metabolic rates are shown by bars beneath the figure. (B) Ash-free dry tissue masses of limpets 3.25 cm in length. Error bars are 95% confidence limits. The ash-free dry tissue mass of a 3.25 cm limpet which has a maintenance metabolic rate (from Parry, 1978) is represented by the solid line, the dashed lines delimit the 95% confidence interval for this estimate.

Unfortunately, many studies which claim to examine "temperature acclimation" or "temperature acclimatisation" cannot be meaningfully interpreted as the activity of ectotherms was not monitored during measurements of metabolic rate (e.g. Marsden, 1979; Armitage and Stinson, 1980; McMahon and Russell-Hunter, 1981). In such studies changes in metabolic rate may result simply from temperature causing changes in activity. Such changes in activity are better interpreted as direct behavioural adaptations to temperature (or some correlated factor) than as metabolic adaptations.

There are two frequently cited, but opposing, views of the adaptive significance of elevated (standard) metabolism at cold temperature. The classical interpretation (Precht *et al.*, 1973; Prosser, 1973) suggests that if standard metabolism was not elevated at cold temperatures it would be insufficient for "maintenance". The alternative view (reviewed by Clarke, 1980; Newell and Branch, 1980) suggests that elevation of metabolism (at any temperature) above that necessary for "maintenance" implies waste, as the energy spent to increase metabolism must be at the expense of somatic and reproductive growth. Hence elevated metabolism should not be expected.

Block and Young (1978) speculate that these conflicting views can be reconciled if we reappraise the meaning of "maintenance" metabolism. They argue that the conflict disappears if the energy required for "maintenance" at cold temperatures is greater than that extrapolated from measurements at high temperatures. In particular, they suggest that the "elevated" metabolism seen during cold acclimation may be the minimum metabolism necessary for maintenance of a viable organism at that temperature in nature. However, the results of this study do not support their interpretation as, although *C. tramoserica* usually has an elevated metabolism in winter, it can survive *in the field* during winter without an elevated metabolism (Fig. 2). While those *C. tramoserica* has survived without an elevated metabolism were caged, it seems unlikely that the cages facilitated the survival of the limpets. Cages have minimal effect on physical conditions (personal observations) and predation on *C. tramoserica* is rare, particularly during winter (Parry, 1982a).

A further hypothesis which explains elevated metabolism in winter is that elevated metabolism is an unavoidable consequence of increased growth rate (Parry, 1978, 1983). The present study provides further support for this hypothesis, as seasonal changes in the metabolic rates of caged and uncaged limpets are consistent with the predictions of an earlier model (Parry, 1978). However, a really adequate test of this model requires that rates of growth and rates of oxygen consumption be measured simultaneously. Unfortunately, rates of growth cannot be measured accurately over the duration of a measurement of oxygen consumption (<2 hr). Consequently, in the analysis that follows I have used the "condition" of limpets as an estimate of the rate of growth. It seems likely that, at least within a season, the fastest growing limpets will be those in the best condition.

Uncaged limpets, except for those collected in August 1980, show similar seasonal changes in metabolic rates to those predicted from the model (Fig. 2(A)), confirming my earlier results (Parry, 1978). This model predicts seasonal changes in the metabolic rate of limpets which have the average monthly rate of growth observed in 1972–1974. A limpet which has a larger-than-average rate of growth would be expected to have a metabolic rate higher than the values predicted in Fig. 2(A).

The higher metabolic rates of uncaged limpets observed in August 1980 compared with August 1982 (Fig. 2(A)) could be explained if the growth rate of limpets collected in August 1980 was approximately twice that of limpets collected in August 1982 (see Fig. 2(A)). Differences in growth rate of this magnitude are feasible as two-fold differences in annual growth between different regions of shore are commonly observed (personal observation).

Furthermore, the better condition of limpets collected in August 1980 compared with August 1982, as indicated by the significantly higher dry tissue mass of limpets collected in August 1980 (Table 1) is likely to be associated with a higher growth rate.

The metabolic rates of caged limpets were significantly lower than those of uncaged limpets in June 1979, August 1980 and August 1982 (Figs 1 and 2). The low metabolic rates of caged limpets are clearly associated with food shortage in cages (e.g. Fig. 2) and similar reductions in metabolic rate are usually observed when ectotherms are deprived of food (e.g. Barnes *et al.*, 1963; Lane and Lawrence, 1979; Klekowski *et al.*, 1979; Quetin *et al.*, 1980).

During June 1979 metabolic rates of caged limpets were significantly below the "maintenance" rate (Fig. 2(A)). These low metabolic rates are associated with a low dry tissue mass (Table 1) and an exceptionally high ash content (Table 1). Furthermore, during the three days following measurements of these metabolic rates, six of the 38 caged limpets died, whereas none of the uncaged limpets died during this period, nor did any limpets die following other measurements of oxygen consumption. Consequently, it seems likely that the metabolic rates of caged limpets during June 1979 are indeed sub-maintenance rates.

During August 1982 the metabolic rate of caged limpets was not significantly different from the maintenance level (Fig. 2(A)). But metabolic rates of caged limpets in August 1980 were higher than those obtained in August 1982 (Figs 1, 2(A)). The higher metabolic rates obtained in August 1980 probably result from these limpets experiencing a shorter period of food deprivation. Limpets were caged prior to the August 1980 measurements for 69 days, whereas they were caged for 89 days in 1982. The better condition of caged limpets in August 1980 can be seen from their higher dry tissue mass (Table 1) and their lower ash content (Table 1). Similarly, in August 1980, the metabolic rates of limpets caged at a density of 10/cage may be higher than those caged at a density of 40/cage, because of the significantly better condition (Table 1, Fig. 2) of limpets caged at the lower density.

The total growth of caged limpets in August 1980 and August 1982 was negative (Fig. 2, Parry, 1982a). However, the metabolic rates observed in August 1980 were probably higher than those in August 1982 because biosynthesis was occurring at a higher rate in August 1980. In the limpet *Patella vulgata* lipid synthesis for gonad development continues at the expense of polysaccaride and lipid reserves even after prolonged (120 days) starvation (Barry and Munday, 1959; Blackmore, 1969). As gonad development in *C. tramoserica* commences in June/July (Parry, 1978) the relatively elevated metabolism observed in August 1980 is probably due to lipid synthesis for gonad development. This interpretation is supported by the higher gonad mass in limpets in August 1980 compared with August 1982 (Table 2). However, gonad mass is a poor measure of the rate of gonad synthesis as the rate of gonad synthesis in limpets (including uncaged *C. tramoserica*) remains essentially constant from the commencement of gonad development to spawning (Parry, 1978, 1982b; Fig. 2(A)).

The similarity of the metabolic rates of caged and uncaged limpets during October 1979 is also probably due to their reproductive condition. During October the "elevation" of the metabolic rate above the maintenance rate is entirely due to gonad synthesis (Fig. 2(A)). It seems likely therefore that although these caged limpets experienced food deprivation (Fig. 2) this did not interfere with gonad synthesis. Stickle and Duerr (1970) (see also Stickle, 1971) also found that starvation of the mollusc *Thais* during gonad development did not cause a reduction in oxygen consumption. Unfortunately, gonads were not dissected from limpets collected in October 1979, but caged limpets collected in October 1982 showed some gonad development (Table 2). Furthermore, the rate of gonad synthesis in October 1979 was almost certainly higher than that in October 1982 as (1) limpets were starved for a much shorter period in 1979 (87 vs 150 days), (2) limpets were caged one month after the usual date of commencement of gonad synthesis in 1979 whereas in 1982 they were caged one month before gonad synthesis commenced, and (3) the total dry tissue mass of a 3.25 cm limpet in 1979 was much higher than that in 1982 (0.23 vs 0.16 g).

Temperature acclimation is known to be influenced by food availability (e.g. Marsden *et al.*, 1973; Newell and Bayne, 1973; Newell *et al.*, 1976) and is eliminated following severe starvation (Barnes *et al.*, 1963; Bayne, 1973; Campbell and Davies, 1975). However, there has been no satisfactory explanation for the

dependence of temperature acclimation on food availability.

In *C. tramoserica* prolonged food shortage causes the elimination of an "elevated" metabolic rate (i.e. "temperature acclimatization") during August but not during October when gonad maturation is proceeding (Fig. 2(A)). My model explains these observations and provides a general explanation for the interdependence of temperature acclimation and food availability. If the elevated metabolic rate observed during "acclimation" is largely the cost of biosynthesis then this elevation must be dependent on food availability.

The present study provides further evidence (see also Parry, 1978, 1983) that changes in the rate of biosynthesis are the major cause of seasonal variation in the metabolism of *C. tramoserica*. More precise data are required before it can be determined whether seasonal changes in maintenance metabolism also occur. However, the present data suggest that most, and possibly all, seasonal changes in standard ectotherm metabolism can be explained without reference to the concepts of temperature acclimation or acclimatisation.

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THE EFFECT OF ENVIRONMENTAL TEMPERATURE ON THE EXCRETION TIME OF SEBUM IN DIFFERENT GENOTYPES OF CATTLE

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Abstract—1. The sebum excretion times (SET) were studied in two breeds of cattle, *Bos taurus* (SH) and the more heat tolerant *B. indicus* (GB).

2. Both breeds were maintained in thermoneutral (24°C) and hot (32°C) environments.

3. SET was defined as the time between synthesis and release of sebum on to the skin surface.

4. The SET was 7 days in SH and 6 days in GB animals at 24°C but was a day earlier at 32°C for each breed.

5. The changing SET with environmental temperature was associated with an increased rate of synthesis of sebum with increased body temperature.

INTRODUCTION

Sebum output in cattle increases with prolonged exposure to hot environments (Smith *et al.*, 1975; O'Kelly and Reich, 1981). Studies on cattle have also demonstrated that total sebum output in thermoneutral and hot environments varies significantly with genotype and coat type (O'Kelly and Reich, 1981) and there are significant genotypes by environment interactions in the composition of sebum excreted onto the skin surface (O'Kelly and Reich, 1982). However, little is known about the mode of secretion of sebaceous glands in cattle. While the sebaceous glands in the skin of most mammals are generally believed to be holocrine one study using histological techniques has suggested that this is unlikely to be so in cattle (Smith and Jenkinson, 1975). The time between synthesis and surface excretion of sebaceous lipids has been reported for man and sheep, whose sebaceous glands are known to be holocrine (Downing *et al.*, 1975, 1977), but not for bovines.

This paper compares the sebum excretion time in two breeds of cattle which differ in their abilities to tolerate heat. The interval of time between intradermal injection of [1-^{14}C]acetate and the appearance of a peak of radioactivity in the wax esters at the injection site was used as the measure of time between synthesis and release of sebum on to the skin surface and was referred to as the sebum excretion time.

MATERIALS AND METHODS

Animals

Four Brahman and 4 British (1/2 shorthorn × 1/2 Hereford) steers, all about 18 months old, were used and the experiment was divided into 2 periods of 16 days each. During the experimental periods the animals were housed in a controlled temperature room. Feed (lucerne hay) was consumed in portions of 3 kg each in the morning and in the afternoon, and water was freely available throughout. The mean body weights (kg ± standard errors) at the beginning of the experiment were: Brahman 225 ± 4; British 281 ± 3.

During the first period a thermoneutral temperature, 24°C, was selected for all 8 animals. In the second period, the temperature of the room was adjusted to 32°C dry bulb, 28°C wet bulb so that the rectal temperatures of the British animals were about 1°C higher than at 24°C and the animals were considered to be suffering consequences of hyperthermia (O'Kelly, 1973). Rectal temperatures and skin temperatures at the sebum collection sites were measured between 10.00 and 11.00 hr with a digital thermocouple thermometer with a short response time (Bailey Instruments, NJ, USA).

Intradermal injections and sebum collection

The skin was prepared for sebum collection before the animals entered the climate room. Hair was removed from a 200 cm^2 area on both sides of the midline in the dorsal thoracico-lumbar region with electric clippers; then the skin was shaved with a safety razor and washed thoroughly with water. One area of 63.5 cm^2 was delineated on both sides of the midline; the area on the left side being matched to an area in the corresponding position on the right side. After three days in the climate room the delineated area of skin was washed thoroughly with *n*-hexane to remove lipid material. Four intradermal injection of 1.25 μCi of [1-^{14}C]acetate solution (Amersham, Australia Pty Ltd) in sterile normal saline (100 μl) were made inside the circular area of 63.5 cm^2 using a tuberculin syringe and a 26 gauge needle; an injection was made in each quadrant of the circle at equidistance from the centre.

For sebum collection, an open glass cylinder of 9 cm i.d. was placed over the marked area of skin. The 63.5 cm^2 area of skin inside the cylinder was then irrigated with 1 × 10 ml followed by 2 × 5 ml of *n*-hexane (Merck, Darmstadt, Germany) using a Manostat pipette. The hexane washings were combined and filtered through paper (Whatman No. 3) to remove skin debris and the filtrate was taken to dryness in a rotary evaporator under nitrogen. The residue was taken up in *n*-hexane and transferred to a tared glass weighing bottle. The *n*-hexane was then evaporated under nitrogen at 55°C to constant weight. The total lipid extract was redissolved in 3 ml of *n*-hexane. Some sebum samples were collected from skin outside the delineated areas.

Extraction and analysis of lipids

An aliquot of the total lipid extract was fractionated into individual lipid classes by thin layer chromatography using glass plates coated with 0.5 mm of Kieselgel G (E. Merck, Darmstadt, Germany) and the 3-stage solvent system of

Table 1. Daily mean rectal and skin temperatures (°C) and weights of the constituents of sebum from British (SH) and Brahman (GB) cattle at different environmental temperatures (°C)

	24°C			32°C		
Air temperature	SH	GB	SE*	SH	GB	SE*
Rectal temperature	38.6	38.3	0.1†	39.4	38.5	0.1‡
Skin temperature	33.7	33.2	0.3	37.9	37.4	0.3
Sebum:						
Sterol esters	787	1226	15‡	1212	2093	73‡
Wax esters	5894	7939	175‡	6558	8761	243‡
Triglyceride	830	1356	25‡	1182	1915	21‡
Free fatty acid	443	459	51	344	353	65
Free sterol	546	720	27‡	904	1178	26‡
Total	8500	111700	200‡	10200	14300	300‡

Values are expressed as $\mu g/100\,cm^2$ of skin surface.
*Standard error of the differences between breeds.
†Breed differences significant at $P < 0.01$.
‡Breed differences significant at $P < 0.001$.

Nicolaides *et al.* (1968). The plates were sprayed with a 0.1% w/v solution of 2,4-dichlorofluorescein in methanol and the lipid bands were visualized under u.v. light. Identification of the lipid bands, except the wax esters, was by co-chromatography with known standards. The quantitation of the lipid fractions, except the wax esters, isolated from the plates was done as described previously (O'Kelly and Reich, 1982). The three fractions which chromatographed between sterol ester and triglyceride bands were designated bands 1, 2 and 3 as described previously (O'Kelly *et al.*, 1980) and the weight of wax esters was taken as the difference between the weight of sebum collected and the sum of the weights of the sterol ester, triglyceride, free fatty acid and free sterol fractions. Recent work by Downing and Lindholm (1982) has identified band 1 as a type I diester, band 2 as a type II diester and band 3 as a triester. As it was difficult to completely separate wax esters 2 and 3 these two bands, both containing 1,2-diols, were eluted together from the plates for the measurements of the incorporation of radioactivity.

Measurement of radioactivity

After preparative thin layer chromatography the individual lipid classes were scraped into scintillation vials containing 1.5 ml of methanol (Merck, Darmstadt, Germany). The vials were shaken and left to stand for 5 min then 15 ml of toluene-fluor containing 4% w/v, of Cab-o-Sil (Packard, Instrument Co.) was added. The vials were capped, shaken thoroughly and after gel formation was complete counted in a scintillation spectrometer (Packard, Downers Grove, IL, USA).

Statistical analysis

The results were subjected to analyses of variance using the method of least squares.

RESULTS

At each environmental temperature there were no significant differences within animals in the daily weight of sebum excreted nor in the weights of the individual lipid classes sampled on days 2, 4, 6, 8 and 10. Consequently, the mean value of the sebum collected from each animal over the 11 day period was used as the measure of the daily sebum excretion and the mean values of the weights of individual lipids excreted on days 2, 4, 6 and 10 are reported in Table 1.

The GB breed maintained a significantly lower rectal temperature than the SH breed at both 24 and 32°C but exposure to the higher environmental temperature significantly increased ($P < 0.001$) body temperature only in the SH animals (Table 1). There were no significant breed differences in skin temperature which was higher ($P < 0.001$) in all animals at 32 than at 24°C.

Between breeds, there were significant differences ($P < 0.001$) in the daily weight of sebum excreted and in the weights of the individual lipid fractions except free fatty acids at both 24 and 32°C (Table 1). In both breeds, the daily weight of sebum excreted and the weights of the individual lipid components of sebum except free fatty acids reported in Table 1 were higher ($P < 0.001$) at 32 than at 24°C.

No labelled lipid material was detected on the skin outside the areas in which isotope was injected. The daily amounts of radioactivity found in the sebum collected from the injection sites were plotted for each animal and comparisons between animals within breeds and treatments showed similar patterns of changes over the 11 day period. There were variations between animals in the total radioactivity recovered, because of differences in the dissipation of the substrate through the dermal vascular system of the injected sites. In order to eliminate the effect of variation in the total amounts of radioactivity recovered the results for each lipid fraction are presented in Figs 1–4 as the daily % of the total counts recovered over the 11-day period.

In the wax ester fractions, the maximum daily output of radioactivity was recovered from the SH animals on day 7 (Fig. 1) and from the GB animals (Fig. 2) on day 6 after injection with [1-^{14}C]acetate at 24°C in contrast to maximum recoveries on day 6 from the SH and on day 5 from the GB animals after injection at 32°C. However, exposure to heat did not alter the times after injection for the recovery of maximum radioactivity in the triglyceride fractions which were on day 5 in the SH animals (Fig. 3(a)) and on day 4 in the GB animals (Fig. 4(a)). In both breeds, the amounts of radioactivity found in the free fatty acids (Figs 3(a) and 4(a)), sterol ester and free sterol (Figs 3(b) and 4(b)) fractions were not significantly different between days at either 24 or 32°C.

The mean amounts of radioactivity found in the wax ester and triglyceride fractions expressed as a percentage of the total radioactivity recovered from the total amount of sebum excreted over the 11 day period are shown in Table 2. At each environmental temperature the percentage incorporation of [1-^{14}C]acetate into wax ester 1 was higher ($P < 0.05$)

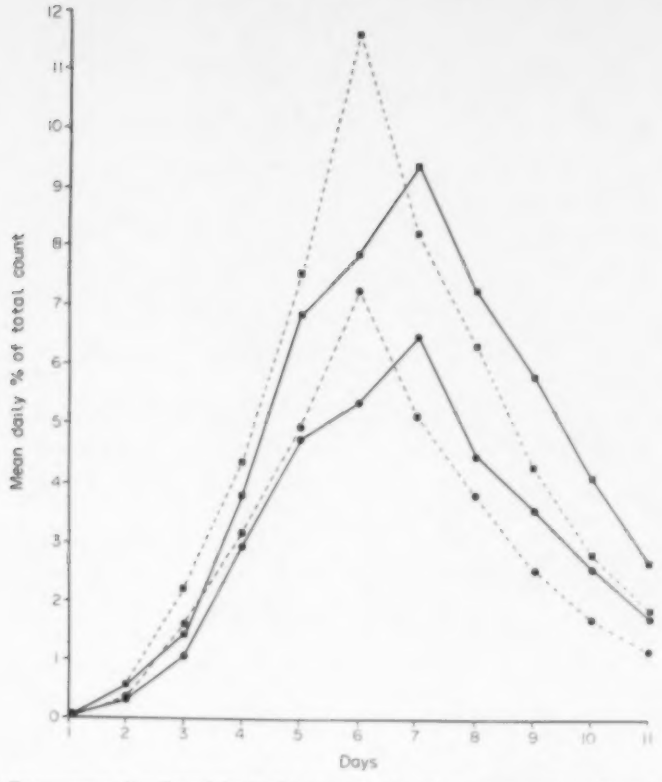

Fig. 1. The daily amounts of radioactivity in the wax ester fractions, expressed as a % of the total counts accumulated over 11 days, of sebum from British cattle at different environmental temperatures. (●) Wax ester 1, (■) wax esters 2 and 3, (——) 24°C, (----) 32°C.

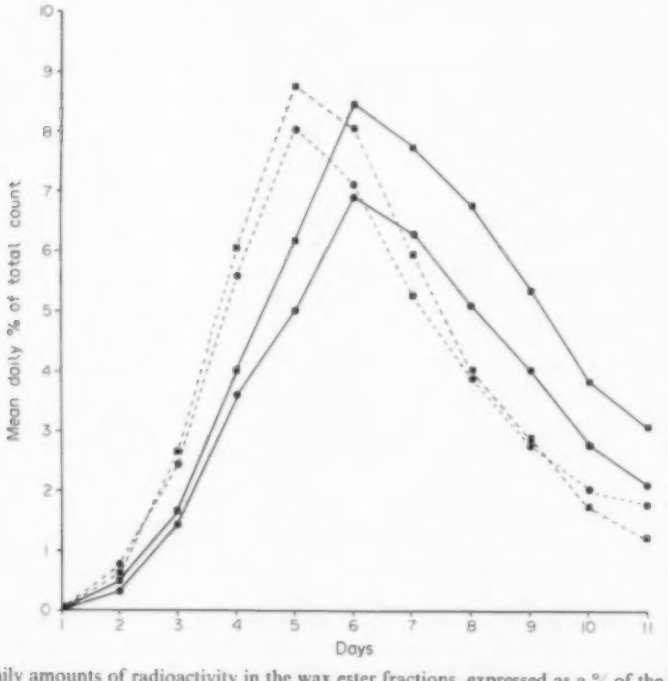

Fig. 2. The daily amounts of radioactivity in the wax ester fractions, expressed as a % of the total counts accumulated over 11 days, of sebum from Brahman cattle at different environmental temperatures. (●) Wax ester 1, (■) wax esters 2 and 3, (——) 24°C, (----) 32°C.

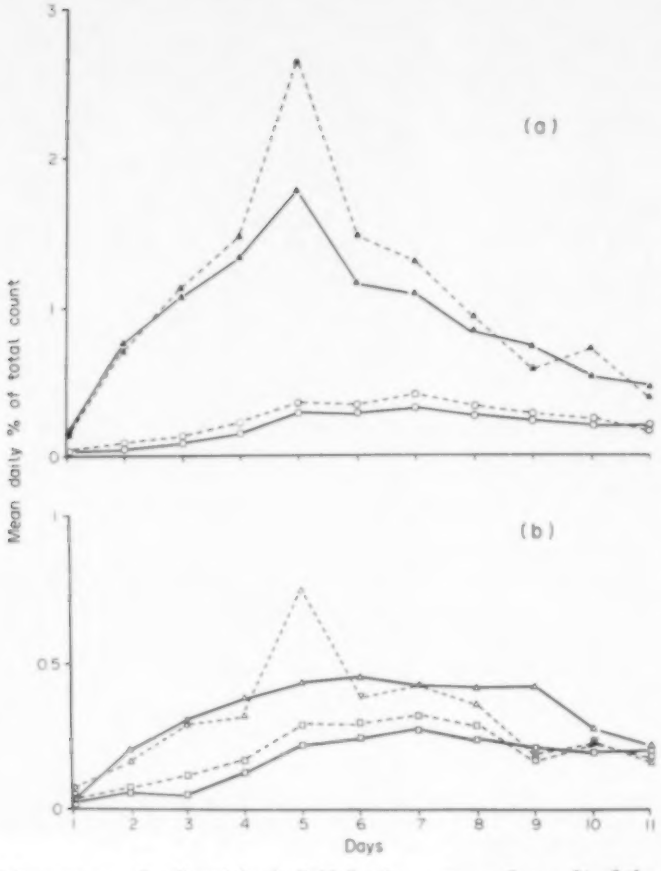

Fig. 3. The daily amounts of radioactivity in lipid fractions, expressed as a % of the total counts accumulated over 11 days, of sebum from British cattle at different environmental temperatures. (——) 24°C, (----) 32°C. (a) (▲) Triglyceride, (○) free fatty acids, (b) (△) sterol esters, (□) free sterols.

in the GB than in the SH breed but at 32°C the percentage incorporation of label into wax ester 2 and 3 was lower ($P < 0.01$) in GB animals than in SH animals. There were no significant differences in the percentage incorporation of label in the wax ester and triglyceride fractions during exposure of SH animals to 24°C or 32°C. However, in the GB breed the percentage incorporation of label was lower ($P < 0.01$) in wax ester 2 and 3 and higher ($P < 0.01$) in triglyceride at 32°C than at 24°C.

DISCUSSION

Labelled lipid was not detected at the uninjected sites and we are of the same opinion as Downing *et al.* (1975) that the [1-^{14}C]acetate was directly incorporated into lipid within a short time after injection. The pattern of changes in the daily amounts of total radioactivity found in the excreted sebum, with over 60% of the labelled lipid emerging over a period of 4 days (days 4–8 after injection of isotope), was similar to that reported for sheep and man (Downing *et al.*, 1975). Each of the major constituents in sebum of sheep and man had a similar excretion time (Downing *et al.*, 1975). However, in our experiments with cattle about 80% of the radioactivity recovered from the sebum was found in the wax ester fractions, a lipid class known to be synthesized exclusively in the sebaceous glands. Consequently, the interval of time between injection of labelled substrate and the peak of radioactivity recovered from the wax esters on the skin surface was considered to be the time taken for sebum to reach the surface after it is synthesized, i.e. the sebum excretion time. However, the physiological concepts of sebum delivery to the skin surface in cattle are little understood. In this study the pattern of changes in the daily amounts of radioactivity recovered after injection of [1-^{14}C]acetate suggests that, as in man and sheep, the sebum is excreted by a holocrine mechanism whereby the lipid laden cells disintegrate and their contents flow through the sebaceous duct onto the skin surface. On the other hand, Smith and Jenkinson (1975) concluded from histological studies that the mode of secretion of cattle sebaceous glands is unlikely to be holocrine.

The sebum excretion time was 7 days in British and 6 days in Brahman animals at 24°C but was a day earlier for each breed at 32°C. However, in each breed the total amount of sebum excreted over the sebum excretion time was independent of environmental temperature. Thus, the British animals excreted 60 mg of sebum per 100 cm^2 of skin surface over 7 days at 24°C and over 6 days at 32°C while the

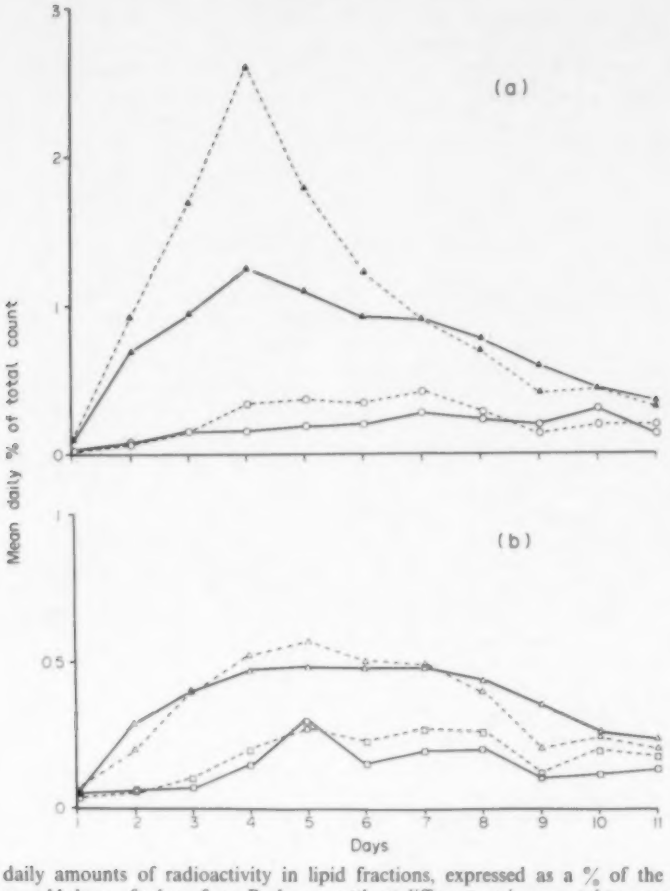

Fig. 4. The daily amounts of radioactivity in lipid fractions, expressed as a % of the total counts accumulated over 11 days, of sebum from Brahman cattle at different environmental temperatures. (——) 24°C, (----) 32°C. (a) (▲) Triglyceride, (○) free fatty acids, (b) (△) sterol esters, (□) free sterols.

Brahman animals excreted 71 mg of sebum per 100 cm^2 of skin surface over 6 days at 24°C and over 5 days at 32°C. The changing sebum excretion time with environmental temperature may then be explained as a consequence of an increased rate of synthesis of sebum with increased body temperature. Blood flow to the skin would increase with rising body temperature in both breeds and would presumably accelerate the supply of metabolites to the sebaceous glands. The breed differences in the production and excretion time of sebum may also be largely explained by differences in blood flow to the skin with associated differences in the rates of supply of substrates to the glands for lipogenesis. It has been proposed that the differences in heat tolerance between these two breeds might be explained partly by differences in the ability to lose heat by non-evaporative pathways (O'Kelly and Reich, 1981). If this is the case, it could be expected that, compared to British cattle at both 24 and 32°C, the blood flow to the skin of Brahman cattle would be higher with an associated higher metabolic activity of the sebaceous glands. However, it is known that endocrine factors regulate the sebum producing activity of sebaceous glands and changing hormonal status on heat exposure could also influence the rate of lipogenesis.

Breed, but not environmental temperature, influenced the times after injection with isotope for the recovery of maximum radioactivity in the

Table 2. Mean amounts of total radioactivity found in the wax ester and triglyceride fractions expressed as a percentage of the total radioactivity recovered from the total amount of sebum excreted over 11 days by British (SH) and Brahman (GB) cattle at different environmental temperatures

	24°C			32°C		
Air temperature	SH	GB	SE*	SH	GB	SE*
Wax ester 1	33.3	37.5	1.6†	31.8	39.2	2.0†
Wax esters 2 and 3	49.7	47.4	1.6	49.7	41.8	1.2‡
Triglyceride	9.7	8.0	1.7	11.1	11.0	1.4

Values are means for 6 animals.
*Standard error of the differences between breeds.
†Breed differences significant at $P < 0.05$.
‡Breed differences significant $P < 0.001$.

triglyceride fractions. In each breed, this was 2 days and 1 day less than the time for the recovery of maximum radioactivity in the wax ester fractions at 24 and 32°C, respectively. This finding suggests that a mechanism exists in the maturing sebaceous cells which restricts the production of triglyceride relative to that of wax esters. In this regard, there is also evidence that during sebaceous cell maturation in humans the quantity of wax esters continues to increase after triglyceride synthesis has ceased (Downing *et al.*, 1977).

The pattern of changes in the daily amounts of radioactivity found in the free fatty acid, sterol ester and free sterol fractions provided no information about the sebum excretion time because the recoveries of radioactivity from these fractions were not significantly different between days. The experimental evidence accumulated by several studies has led to the hypothesis that the free fatty acids of human skin surface lipids are largely derived from sebum triglycerides mainly through the lipolytic action of microbial lipases (Shalita, 1974). While there is evidence that in cattle the sebum triglycerides contribute free fatty acids to the skin surface lipid layer (O'Kelly and Reich, 1982) it is also likely that some are derived from degradation of phospholipids in the keratinizing epidermis (Long, 1970). The fatty acids liberated from the phospholipids may become esterified to sterols (Nikkari and Valavaara, 1970) and it is considered that the esterified sterols of cattle surface lipids are mainly of epidermal origin (O'Kelly and Reich, 1982). Although it is generally assumed that, in the skin surface lipids of animals, the free sterol originates from the epidermis there is evidence that a significant portion of this lipid component originates in the sebaceous glands of cattle (O'Kelly and Reich, 1982). Thus, the daily amounts of radioactivity recovered from these three lipid fractions would represent variable contributions from both the sebum and epidermis and would not be expected to show the same patterns as those lipid components arising solely from the sebaceous glands. It is noted that in a study of the time course of lipid formation in sebaceous glands Downing *et al.* (1975) reported that the variations in the specific radioactivity of the free fatty acids of human skin surface lipids and in the intensity of isotopic labelling of free sterol and sterol esters in sheep surface lipids followed those of their respective major lipid constituents.

The skin temperature of all animals was about 4°C higher in the hot (32°C) than in the thermoneutral (24°C) environment. However, while the Brahman cattle maintained nearly normal rectal temperatures at 32°C the British animals were suffering hyperthermia, which is known to cause derangements in lipid metabolism. For example, hyperthermia in cattle induced by prolonged exposure to high environmental temperatures lowered the plasma concentrations of free cholesterol, cholesterol esters, phospholipid and lecithin–cholesterol–acyl transferase activity and increased the amount of fat excreted (O'Kelly, 1973; Noble *et al.*, 1973). In the Brahman animals the percentage incorporation of total radioactivity was lower in wax esters 2 and 3 and higher in the triglyceride fractions of sebum collected at 32°C than at 24°C (Table 2). On the other hand, the amounts of total radioactivity incorporated into sebum which were distributed between wax ester and triglyceride fractions were not significantly different in British animals at 24°C and at 32°C. These findings suggest genetic differences in the biochemical processes involved in acclimatization to hot environments.

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THERMOREGULATORY BEHAVIOR OF THE TURTLE, *PSEUDEMYS SCRIPTA ELEGANS*, IN A THERMAL GRADIENT*

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Abstract—1. From a given starting temperature, turtles were allowed to move freely within an experimental thermal gradient. Temperatures were measured from the surface of the plastron (T_a) and near the heart (T_{heart}). From these temperatures the median selected temperature (MdST) and the mean selected temperature (MnST) were calculated.

2. MdST and MnST, calculated from T_a or T_{heart}, are positively correlated with time of day between 07:00 and 17:00 hr with slopes ranging from 0.42°C hr^{-1} to 0.60°C hr^{-1}.

3. The calculated regression equations for the three turtles tested do not differ significantly in slopes at the 95% level.

INTRODUCTION

A criterion for thermoregulatory behavior in ectotherms is the mean selected temperature (following the terminology of Pough and Gans, 1982) of animals moving freely in a thermal gradient. DeWitt and Friedman (1979) proposed the median selected temperature (MdST) rather than the mean selected temperature (MnST) to characterize the central tendency of body temperatures in ectotherms, because the distribution of body temperatures in these animals tends to be negatively skewed.

Diel rhythms of the MnST are known for fish (Reynolds *et al.*, 1978 a,b; Reynolds and Casterlin, 1979) and lizards (Regal, 1967, 1974; Cowgell and Underwood, 1979). For freshwater turtles such a rhythm could not be detected for the MnST (Graham and Hutchinson, 1979; Crawshaw *et al.*, 1980). The temperature tolerance, however, does show a diel rhythmicity (Kosh and Hutchinson, 1968).

The purpose of this study is to test whether this lack of a diel cycle might be attributed to experimental or statistical procedures, and to examine the time course of thermoregulatory behaviour more closely.

METHODS AND MATERIAL

The turtles were obtained from private holders and kept in 120 l. glass aquaria equipped with plastic platforms for occasional emergence. Air and water temperatures were held constant at 23 ± 1°C by an air condition system. Illumination (700 lx) was provided by fluorescent lights at the ceiling running parallel to the gradient apparatus. They were connected to a time switch set at a photoperiod of 14 hr light and 10 hr dark, centered at 12:00 hr Central European Time. The turtles were subjected to this light–temperature regime for at least 2 weeks prior to the experiments to allow complete acclimation (Hutchinson and Kosh, 1964). They were fed canned cat food and raw beef liver (*ad lib.*) twice weekly. Turtle C did not feed during the experimental period (14 January 1983–17 February 1983) and for at least 2 weeks prior to the experiments. All three turtles were females weighing 285, 499 and 1186 g at the time of the experiments (October 1982 through February 1983). A total of 590 experiments were carried out between 07:00 and 17:00 hr (Central European time).

Gradient apparatus

The gradient apparatus (Fig. 1) consisted of a plastic trough, 300 cm long, 30 cm wide and 24 cm deep. At the bottom it was divided into six compartments of equal size by five partitions (a) 5 cm high. Each compartment was equipped with copper tube windings (b), through which thermostats pumped water of different temperatures, increasing by about 10°C from one compartment to the next. A perforated plastic tube (c), with two side branches (d) in each compartment, ran along each side of the gradient and was connected to an aquarium pump on either end. Plastic tube as well as copper tube windings were covered by a perforated metal floor (e) on which the turtles could move freely. The water level was kept about 12 cm above this floor. The air bubbles rising from the plastic tube reduced the thermally generated overall water circulation so that a temperature difference of about 15°C between both ends of the gradient could build up. The temperature on the cold side normally ranged from 18 to 24°C and on the warm side from 34 to 40°C.

Temperature measurements

For the recording of deep body temperature a metal tube was inserted 1 cm through a 1 mm hole drilled in the anterior-medial corner of the right abdominal scute and fixed with Histoacryl® (Braun–Melsungen). Into this tube, which was sealed on its inner end, a thermocouple (Ni–CrNi) could be inserted during the experiments to measure the temperature near the heart (T_{heart}; Spray and May, 1972; Spray and Belkin, 1972, 1973). A second thermocouple measured the ambient temperature at the surface of the plastron (T_a) to control the location of the turtle in the gradient. The thermocouple wires were secured to the body with tape. The free thermocouple wires, which connected the tested turtle to a Philips comprecorder (PR 3500) in the adjacent room, were loosely suspended from a point 2 m above the middle of the gradient and did not inhibit the turtle's mobility. T_{heart}, T_a as well as the highest

*This paper forms part of a dissertation of the first author to be submitted in partial fulfillment of the requirements for the degree of Dr rev. nat. in Biology at the University of Hamburg.

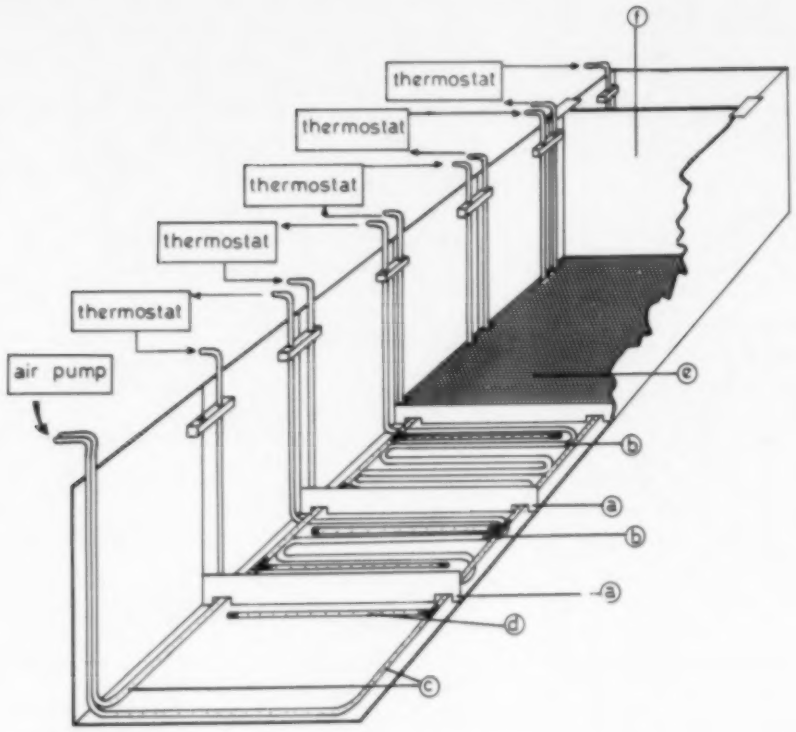

Fig. 1. The gradient apparatus. (a) Partitions (5 cm high) dividing the bottom of the trough into six compartments, (b) copper tube windings (not shown in first compartment), (c) perforated plastic tube with side branches (d), (e) perforated metal floor (only shown in posterior part of the trough), (f) one of two partitions used to restrict the turtle to one of the six compartments.

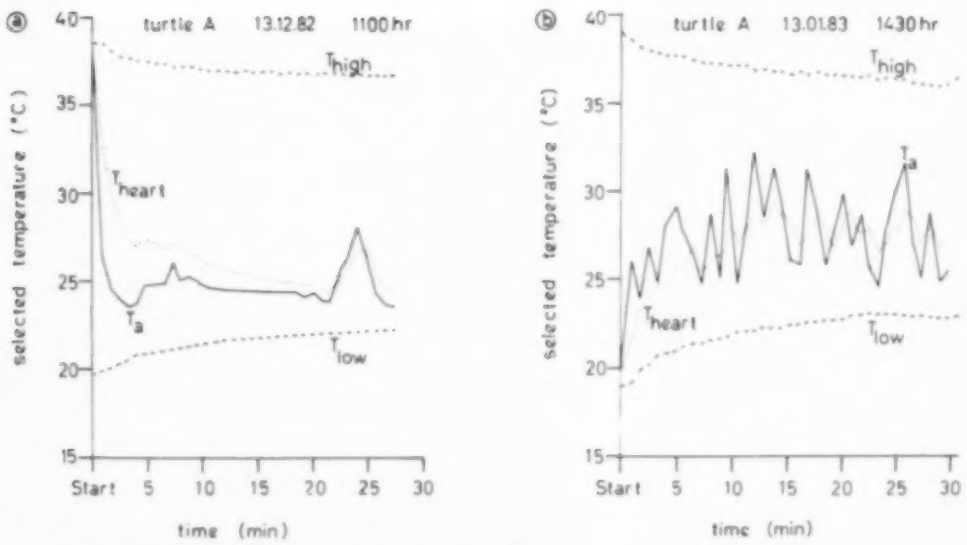

Fig. 2. Time courses of two experimental runs of turtle A. T_{heart} = temperature near the heart, T_a = temperature at the surface of the plastron, T_{high} = highest temperature in the gradient, T_{low} = lowest temperature in the gradient. (a) The turtle moves almost directly to a certain temperature and stays there for a relatively long time. (b) The turtle moves into a temperature range and oscillates around the midpoint of this range.

(T_{high}) and lowest temperature of the gradient (T_{low}) were automatically printed by the recorder at 48 sec intervals. The resulting graphs (Fig. 2) were fed into a computer by means of a graphic table and stored on magnetic discs.

Half an hour before the experiment was started the turtle was confined to one of the six compartments by introducing a partition (Fig. 1 (f), 19 cm high) on each side so that at the beginning of the experiment T_{heart} equalled T_a in that compartment. The starting temperature was almost the same for all experiments carried out during one day. From day to day starting temperature was changed in increasing and decreasing order over the range of 12–41°C. Each experimental run lasted between 20 and 30 min. The heart temperatures from the tenth minute until the end of the experiment were used to calculate the MdST and the MnST. The same calculations were carried out on T_a from the tenth minute until the end. The first 10 min had to be disregarded, because T_{heart} and T_a during that time were influenced by the starting temperature (Fig. 2), which was forced upon the turtle and not selected.

Statistics

Analysis of covariance and the Newman–Keul multiple-range test were applied for comparing slopes and elevations of calculated regression equations (see Zar, 1974).

RESULTS

Figure 2 shows two examples of time courses of thermoregulatory behavior of *Pseudemys scripta elegans*. The turtle may either move directly to a certain temperature and stay there (Fig. 2a) or it may select a temperature range and oscillate around the midpoint of this range (Fig. 2b). The type of behavior is not characteristic for the individual turtle and is independent of the starting temperature. The turtles may show any combination of this two extremes of thermoregulatory behavior, in any case it does not take more than 10 min before T_{heart} reaches the selected temperature range. The reduction of the temperature difference in the gradient ($T_{high} - T_{low}$) is mainly caused by removing the partitions that confined the turtle to a certain compartment, and to a lesser degree by the movements of the turtle itself.

In Fig. 3 the MdST (calculated from T_{heart}) of turtle A is plotted versus time of day. Between 08:00 and 17:00 hr the data points form a highly significant regression ($P < 0.001$). The calculated regression lines for all three turtles are also presented in Fig. 3. They do not differ significantly in slopes (b: 0.45–0.59) at the 95% level ($0.1 < P < 0.25$), whereas the elevation of the regression line for turtle B is significantly different from the elevations of the regression lines for turtles A and C at the 95% level. The latter two are not significantly different at this level.

The correlation with time of day is not very different for the MdST and the MnST, whether they are calculated from T_{heart} or T_a (r: 0.35–0.58). Although T_a is more variable than T_{heart} (see Fig. 2), the correlation coefficients are still highly significant ($P < 0.001$). The slopes of all twelve calculated regression lines range from 0.42°C hr^{-1} to 0.60°C hr^{-1}.

DISCUSSION

It is known that *P. scripta elegans* selects a specific temperature when given the choice in a thermal gradient (Gatten, 1974; Crawshaw *et al.*, 1980), but it has been denied that the mean selected temperature (MnST) might be subject to diel variations (Crawshaw *et al.*, 1980). The present study shows that in fact there is a diel rhythmicity underlying the temperature selection of *P. scripta elegans*, which can be approximated by a linear regression between 07:00 and 17:00 hours (Fig. 3). This linear regression is hardly influenced by the method used to calculate the central tendency of selected temperatures, indicating that the distribution of selected temperatures does not deviate significantly from a normal distribu-

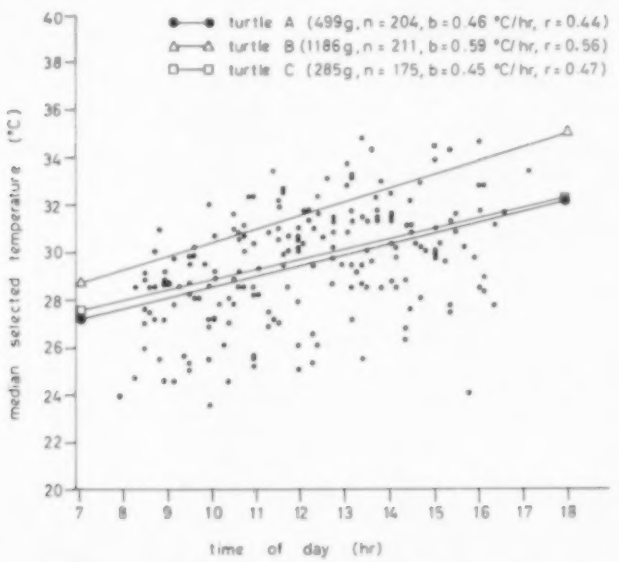

Fig. 3. Median selected temperatures of turtle A (calculated from T_{heart} from the tenth minute until the end of the experiment) plotted versus time of day. Each point represents one experiment. Straight lines: calculated regression equations for turtles A, B and C.

tion. The MdST and the MnST show a highly significant ($P < 0.001$) correlation with time of day between 07:00 and 17:00 hours, whether T_a or T_{heart} is used for the calculations, so that the statistic employed cannot be the reason for the lack of diel variation in the experiments of Crawshaw *et al.* (1980), who calculated the MnST from T_a.

Graham and Hutchinson (1979) suspected that the lack of diel variation in their experiments with *Chrysemys picta*, *Clemmys guttata*, and *Sternotherus odoratus* might have been due to fasting of the turtles. Turtle C in the present study, however, was certainly in a fasted status, and still the regression lines did not differ significantly from that of the well fed turtle A (Fig. 3).

If neither the nutritional status nor the method employed can prevent or mask the diel rhythm of temperature selection in *P. scripta elegans*, the most probable explanation for the absence of this rhythmicity in the experiments of Crawshaw *et al.* (1980) is the relatively small number of their data points: they tested two turtles for 3 days each, resulting in 60 values of MnST per turtle for 30 min intervals between 07:00 and 17:00 hr (compared to 175–211 values per turtle in the present study). Considering the variability of thermoregulatory behavior in *P. scripta elegans*, this seems to be an insufficient number to detect the diel cycle.

Crawshaw *et al.* (1980) report that within 40 min the majority of the turtles were already at temperatures similar to those occupied by the animals living continuously in the gradient. In the present study the turtles were much faster, reaching the selected temperature range in about 10 min (Fig. 2). The reason might be the cold-acclimation (3°C) and the low starting temperature (9°C) in the experiments of Crawshaw *et al.* (1980), which tend to slow down the turtles' reactivity.

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THE EFFECT OF TEMPERATURE–SALINITY COMBINATIONS ON OXYGEN CONSUMPTION OF THE TROPICAL GASTROPOD, *MUREX POMUM*: A RESPONSE-SURFACE APPROACH

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Abstract—1. The respiratory behaviour of *Murex pomum* was monitored for combinations of salinity and temperature, encompassing a salinity range of 25–40‰ at 3‰ intervals and temperatures of 20, 23, 27, 32, and 35°C.

2. Respiratory rates were determined to be influenced by salinity, temperature and the salinity–temperture interaction.

3. High temperatures tended to increase salinity dependent variation in oxygen consumption rate, while low temperatures reduced them.

4. High salinity tended to increase temperature dependent variations in respiratory rate, while low salinities reduced them.

5. No clear pattern of Q_{10} values emerged from the data.

6. Response surface contour maps and attendant model equations were presented in addition to the traditional graphical plots.

INTRODUCTION

The increasing concentration of anthropogenic activities on coastal regions is severely taxing the carrying-capacity of many individual ecosystems. The optional balance point between development and environmental health can be signalled by organisms well adapted to the region and whose response systems are known over a wide enough range so that they act as indicators of environmental stress. In this study, the gastropod mollusc *Murex pomum* was chosen as an environmental indicator.

The respiratory behaviour of the adults of the gastropod mollusc *Murex pomum* was examined under conditions of single factor variations—temperature and salinity—and their responses quantified in terms of weight-specific oxygen consumption by Sander and Moore (1978, 1979). The effect of these factors on the embryology and larval development was also investigated (Moore and Sander, 1981) in an attempt to elucidate the plasticity of the species under these circumstances. The simple empirical results obtained from these experiments were quite easily expressed in the linear response score (Y) on the levels of one of the variables (x_1). The derived equations were:

$$Y = b_0x_0 + b_1x_1 \text{ or } O_2 = aW^b$$

expressed logarithmically as:

$$\log O_2 = \log a + b \log W.$$

However, it is recognized that environmental changes are very often multifactorial and that the level of response to any one factor will be tempered in a positive or negative way by other changes occurring simulataneously. For example, ambient salinity is itself modified by temperature and it is conceivable that two or more factors acting in concert exert a compounded influence on the physiological response system. As pointed out by Alderdice (1972), when the response of an organism is considered over levels of two variables (x_1 and x_2) the straight line relationship becomes a surface or plane in x_1, x_2. Furthermore an interaction term, $b_{12}x_1x_2$, may enter the expression. The magnitude of the response (Y) may change over levels of x_1 in a manner dependent on the level of x_2 at which Y is obtained.

The expression then becomes:

$$Y = b_0x_0 + b_1x_1 + b_2x_2 + b_{12}x_1x_2$$

and the straight line relationship derived earlier may be considered as a section or transect of the plane.

The effects of salinity–temperature combinations on the aerobic metabolic rates of various marine invertebrates (mostly Crustacea) are well documented (e.g. Dehnel, 1960; McFarland and Pickens, 1965; Van Winkle, 1968; Engel and Angelovic, 1968; Gilfillan, 1972; Dorgelo, 1973; Bulnheim, 1974; Dimock and Groves, 1975; Simmons and Knight, 1975; Nelson *et al.*, 1977; Stern and Achituv, 1978).

Response surface methodology, first introduced by Box and Wilson (1951), can provide a comprehensive insight into the inter-relationship of biological and ecological phenomena and, consequently, there has been increasing application of response surface contour plots in presenting multifactorial effects of environmental parameters such as salinity and temperature on biological mechanisms and reactions. Of these, larval development and survival have been most commonly studied (e.g. Haefner, 1969, 1970; Cain, 1973; Lough and Gonor, 1973; Hicks, 1973; Lowthion, 1974; Kennedy *et al.*, 1974; Hrs-Brenko *et al.*, 1977; Paul, 1980; Tettelbach and Rhodes, 1981).

This study of the combined effect of temperature and salinity on the tropical marine gastropod *Murex pomum*, represents a second order experiment aimed primarily at determining the response of the species in conditions of dual stress and secondly at understanding the combined effects of the above factors on the functional capacities of the organism, that is the inter-relationship between the biological response (respiration rate) and the coincident impact of variations of the two environmental factors. An attempt will also be made, within the compages of response-surface methodology, to determine if possible, the quantitative value of the interaction of the two factors.

MATERIALS AND METHODS

As in the earlier experiments in this series, specimens of *M. pomum* collected off Young Island in St Vincent, West Indies, were transferred to the Bellairs Research Institute in Barbados and there maintained in good physiological health in conditions closely simulating their natural environment.

A 5 × 6 factorial experiment was set up to cover the range of 20–37°C temperature and 25–40‰ salinity. These ranges in physical parameters were the ones previously used as unifactorial variables as it was hoped to establish a good basis for comparison in the response dimensions. Again, as in earlier experiments, respiration was used as the measurable response.

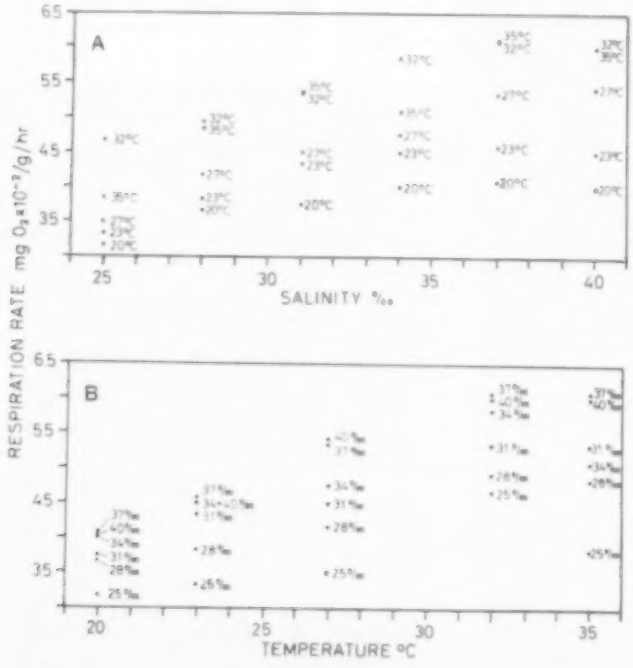
Prior to the experimentation, the snails were sorted and matched for size so that each group, containing 15 adults, had approximately the same weight distribution and average weight. This removed any bias due to size. One group of 15 animals was acclimatized to each treatment condition for a 24 hr period in a water bath, at the end of which respiratory performances were measured individually for a 1 hr period so that for the 30 different combinations of temperature and salinity, 450 measurements were taken. Experimental techniques used were those described by Sander and Moore (1978, 1979) and involved measurement of the initial and final oxygen tension in the respiratory medium with a Beckman Field Analyser O_2 probe, and calculation of the weight-specific oxygen consumption. The results were analyzed with the aid of Statistical Analysis System (SAS) package programs.

RESULTS

Figures 1(A) and (B), showing the mean metabolic rate of 15 animals at each temperature–salinity combination, indicate definite trends of respiratory activity with changing salinity and temperature. Each plot shows respiration rate means increasing simultaneously with increasing salinity and temperature, leveling roughly at 37‰ and 32°C respectively. These circumstances suggest strong correlations between temperature and respiration, and salinity and respiration. Pearson correlation coefficients, measuring correlation between group respiration means and the corresponding group salinities ($r = 0.6053$; $P < 0.001$) and group temperatures ($r = 0.7046$; $P < 0.01$) corroborate this. Also, determination of correlations between all individual respiration rate values and their corresponding salinity ($r = 0.3893$; $P < 0.001$), temperature ($r = 0.4531$; $P < 0.001$) and product (salinity × temperature) ($r = 0.6041$; $P < 0.001$) values indicate that each of the three factors can, through a simple linear relationship, account for variations in respiration.

Regression analysis was performed utilizing again the three factors—salinity, temperature and their product—as independent variables accounting for variation in the dependent variable, respiration rate. This regression results in the model (A)

Respiration rate $= 0.0248 - 3.9278 \times 10^{-4}$, tem-



Figs 1A and B. Mean respiratory rate of 15 specimens of *Murex pomum* at each temperature and salinity plotted against salinity (A) and temperature (B) respectively.

perature -2.3983×10^{-4}, salinity $+ 4.5080 \times 10^{-5}$ (temperature × salinity).

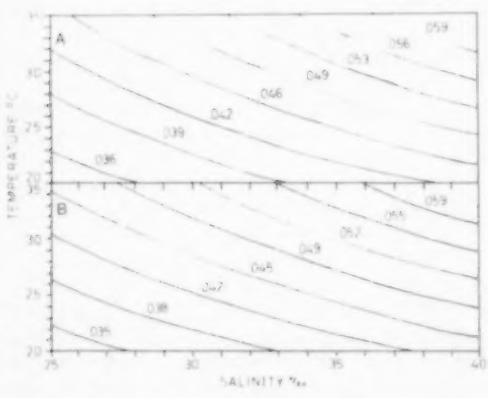
The r^2 value associated with this model indicates the level of agreement on a scale of 0.0 to 1.0 between the respiration level predicted by the model and the actual respiration values. The r^2 value of 0.3663 ($r = 0.6052$) suggests high predictability of the model. The latter is highly significant at greater than the 99% level as indicated by a relatively high associated F value (85.93; d.f. 3). This high significance is due to the large number of observations and the significant difference between group means. The high predictability is apparently due to the low within group variability the data exhibit.

Because of the indication shown earlier of a strong interaction effect between salinity and temperature, it was decided to perform a further regression analysis utilizing the single factor (salinity × temperature) as the single independent variable accounting for variation in respiration rate. The regression results in the model (B)

Respiration rate $= 0.0158 + 3.4421 \times 10^{-5}$ (temperature × salinity).

This model gives an r^2 value of 0.3650 ($r = 0.6042$) indicating a similar strong agreement between actual and predicted respiration rates. The large F value (257.47; d.f. 1) indicates that the model is highly significant ($P < 0.001$). The results suggest that the simplest adequate model could be one including only the temperature × salinity product as the independent variable. The fact that the interaction term is strongly correlated to each of the two main effects, salinity ($r = 0.6105$; $P < 0.001$) and temperature ($r = 0.7824$; $P < 0.001$), minimizes its contributory value in model (A).

Figures 2(A) and (B) show response surfaces determined from models (A) and (B) respectively. Both of these models adequately account for the real trends apparent in the data and it can be seen by comparison, that there is marked similarity of values at the measured contours. Any further refinement of these models would primarily depend on reduction of within group variability or studies concentrating on a more restricted portion of the temperature and salinity curves.



Figs 2A and B. Response-surfaces depicting contour plots of respiratory rates of *Murex pomum* along salinity (x) and temperature (y) gradients based on models A (A) and B (B).

DISCUSSION

The statistical treatment of the experimental data presented in Results indicate significant effects of simultaneous action of salinity and temperature on respiration of *M. pomum*, including a considerable degree of single factor salinity and temperature dependence. Over the response area considered it can be noted that the patterns of response to changing temperature or salinity, when one of these was controlled to approximate ambient level i.e. 27°C or 34‰ (Figs 1A and B), are consistent with those elicited previously from this organism (Sander and Moore, 1978) insofar as the respiratory rate increased directly with increases in temperature and salinity over broad ranges of these parameters. Similarly the same points of inflexion (maximal oxygen consumption) at roughly 32°C and 37‰ signify a decrease or levelling of the rate of oxygen uptake. The mechanisms which mediate such responses have been reviewed by the authors (Sander and Moore, 1978, 1979). These same general patterns of response were repeated above and below ambient salinity and temperature, suggesting persistent and consistent effects of these two components of the marine environment (Figs 1A and B).

It was a principal object of the present study however, to examine the interaction of salinity and temperature on respiratory rates. This may be accomplished by studying the spectra of variations in respiratory rates. The results, including statistical proof of a significant effect of the salinity–temperature interaction, may be interpreted from visual inspection of Fig. 1 and Fig. 2 but benefit from further quantification. Tables 1, 2 and 3 have been compiled to this end.

Table 1. Absolute and relative (‰) increases in oxygen consumption rates (mg O_2/g/hr) at 37‰ compared to 25‰ for the five experimental temperatures

Temperature (°C)	35	32	27	23	20
Respiratory rate increase	0.0227	0.0145	0.0187	0.0125	0.0090
Per cent increase	59	31	54	38	28

Table 2. Absolute and relative (‰) increases in oxygen consumption rates (mg O_2/g/hr) at 32°C compared to 20°C for the six experimental salinities

Salinity (‰)	40	37	34	31	28	25
Respiratory rate increase	0.0194	0.0200	0.0179	0.0155	0.0115	0.0145
Per cent increase	48	49	45	41	31	46

Table 3. Q_{10} values at six experimental salinities and five ranges in temperature

	35–32°C	32–27°C	27–23°C	23–2)°C	32–20°C
40‰	1.01	1.20	1.63	1.43	1.39
37‰	1.02	1.30	1.47	1.48	1.40
34‰	—	1.49	1.15	1.47	1.36
31‰	1.01	1.42	1.08	1.60	1.33
28‰	—	1.43	1.23	1.09	1.28
25‰	—	1.78	1.11	1.18	1.43

Figure 1 suggests that *M. pomum* conforms to the respiratory behaviour of many invertebrates as noted by Kinne (1971), insofar as "supranormal temperatures tend to increase salinity dependent variation in metabolic rate, while subnormal temperatures frequently reduce them". This pattern is also exemplified in *Artemia* (Engel and Angelovich, 1968) and *Euphausia pacifica* (Gilfillan, 1972) but an opposite effect has been demonstrated for *Neomysis intermedia* (Simmons and Knight, 1975). Similarly, high salinities induced a greater respiratory response pattern to changes in temperature than did lower concentrations. Mixed results were obtained for *E. pacifica* by Gilfillan (1972) (consequently the salinity–temperature interaction factor was not statistically significant), and an opposite effect was found for *N. intermedia* (Simmons and Knight, 1975) and *Macrobrachium rosenbergii* (Nelson *et al.*, 1977).

The above observations refer to absolute values, but it is a point of interest whether it is more appropriate to use a relative rather than an absolute scale in interpretation of the results. Tables 1 and 2, comparing differences in respiratory rates between lowest salinity (25‰) and lowest temperature (20°C) and the inflexion points (37‰ and 32°C respectively), address this matter. In this it can be seen that the numerical order of absolute values for 32°C and 23°C are reversed for the relative values (percent increase) in Table 1, although the general pattern (a rough gradient) remains the same. This is not the case in Table 2, where the gradient of absolute values is not repeated for the relative numbers. Q_{10} values, calculated from an exponential equation and shown in Table 3, confirm this. The last column, giving Q_{10} values for the response of the effective metabolic rate to the experimental 32–20°C temperature range at various salinities, depicts no gradient.

No clear composite picture of Q_{10} values emerges from the complexity of interactions effected by the two components, salinity and temperature. The numbers listed in Table 3 are expectedly low for the highest temperature range but are variable for intervals below 32°C. As a result, it is difficult to define or surmise the actual effect the significant salinity–temperature factor has on the biological mechanisms of *M. pomum*. The difficulty arises from the fact that at no time during the series of investigations was either parameter a truly unifactorial one; that is at no time was either of these values zero, and inasmuch as the lethal lower limits for this organism was established to be 11°C and 11‰ respectively, it is not scientifically feasible to extrapolate to the zero point in either of the models. The extent to which the one factor influences the effect of the other on a biological function can possibly best be studied in an organism whose tolerances include the zero point of both. In so far as *Artemia* is one such organism, the results of Engel and Angelovich (1968) might be very significant, and it is of some interest that the respiratory behaviour of *M. pomum* is comparable to that of *Artemia* as demonstrated by these two investigators. The immeasurability of the interaction factor inherent in working with organisms from a saline environment probably explains why the literature does not provide satisfactory explanations for all cases of different types of respiratory response to salinity described for both steno- and euryhaline organisms such as are reported in the reviews of Wikgreen (1953), Kinne (1964) and Tweede (1965).

What can be identified and expressed mathematically is the response surface for the species—as shown in Figs 2(A) and (B). The fundamentals of the response surface methodology employed are discussed in many papers (e.g. Hill and Hunter, 1966; Lindsey *et al.*, 1970) and a number of textbooks (perhaps the most comprehensive is still that given in a book written by a group of chemists, engineers and statisticians and edited by Davies (1954)) but suffice to say that in the present case it enables the reader, studying the surface in the form of contours, to gain a quick visual appreciation of the general relationship between the two variables and the response. As such, the 2-factor response surfaces in Figs 2(A) and (B) readily indicate (1) a direct relationship between increasing weight-specific respiration of *M. pomum* and concurrently increasing salinity and temperature, and (2) a slower rate of effect of salinity and of temperature at low temperature and salinity respectively—as illustrated by the broader intervals between contour lines at the lower end of both scales. Conversely, as salinity and temperature increased, the rate of response increased.

Both models derived are highly significant and the attendant equations render good predictability of *M. pomum's* respiratory rates as a function of the two variables considered; but by smoothing the surfaces from limited data the models necessarily lose some accuracy. This inevitable "lack of perfect fit" is perhaps most apparent in the region of high salinities and temperatures beyond the approximate inflexion points of 32°C and 37‰. Collection and employment of additional data in this region (ideally to lethal limits) in conjunction with the extension to a higher order polynomial in the calculations, would better account for curvature in the response surface and fully (i.e. through 360°) define the peak with concentric ellipses.

In respect of biological inter-relationships, it is a basic fact that "metal ions play a prominent role in biochemical/physiological phenomena, resulting in part from their association with both large and small molecules, including metal ion–protein complexes" (Mahler and Cordes, 1969). It might be expected that the salinity concentration of the respiratory medium will affect the state of association with such molecules and complexes. In the case of metallo-proteins such ions are an integral part of protein structure and their removal inevitably leads to destructuring of the molecule. In addition there are a large number of reversible metal ion–protein interactions which lead to the stabilization of a particular molecular conformation. Such a case is exemplified in the obligatory association of ATP with metal ions before this molecule can react in ATP-dependent enzymatic reactions.

The effects of temperature on reaction kinetics is too well known to warrant discussion, but the two of greatest probable pertinence here are its effects on the mobility of ions and also its effect on the structure of substrate and agent molecules. To the extent that temperature affects the rate of metal ion–protein interactions, or the state of stability of protein configuration, it interacts with salinity to affect respi-

ratory rate, as respiration will decrease when the respiratory particles no longer possess the requisite degree of structural integrity. It is also of note that the upper point of inflexion on the oxygen consumption curve probably marks the point at which the exothermic reactions in the respiratory chain are blocked by heat perfusion of the system.

In conclusion, it appears that the interaction of the effects of salinity and temperature presented in this study is a manifestation of a general property of *M. pomum*. Detailed studies on the biology and behaviour of this gastropod in its native habitats would serve to provide an ecological context for the interpretation of the respiratory responses to environmental conditions, and would aid not only in elucidating the complexity of the salinity–temperature induced responses, but also in predicting the mechanisms which govern these responses.

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COMPARATIVE MORPHOLOGY AND CHEMICAL CONTENTS OF MALE MANDIBULAR GLANDS OF SEVERAL *CENTRIS* SPECIES (HYMENOPTERA: ANTHOPHORIDAE) IN COSTA RICA*

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Abstract—The morphology and chemical contents of male mandibular glands from several species of Costa Rican *Centris* bees are described. Species that marked their territories with oral secretions had enlarged mandibular glands containing varying amounts of known monoterpenes. Species that marked territories with hindleg secretions and those that are not known to chemically mark territories had vestigial mandibular glands containing little or no volatile oil. With regard to territorial marking behavior, chemo-ecological and phylogenetic relationships of several *Centris* subgenera are discussed.

INTRODUCTION

Males of many species of *Centris* bees establish mating territories (Frankie and Baker, 1974; Raw, 1975; Alcock, 1976; Frankie *et al.*, 1980). Some of these males mark their territories with scents produced by mandibular glands (Raw, 1975; Frankie *et al.*, 1980; Vinson *et al.*, 1981) or hindleg glands (Raw, 1975; Williams *et al.*, 1983), whereas others set up and maintain territories without chemical marking (Frankie and Baker, 1974; Raw, 1975; Frankie, Vinson and Coville, unpublished observations).

Frankie *et al.* (1980) described the role of mandibular gland scents in the mating system of *Centris adani* Cockerell. Males of this species establish territories primarily in dry grassy habitats, which are not apparently associated with nesting sites or food plants. Mandibular gland secretions are deposited on vegetation that defines their territories, and sexually receptive females are believed to locate males by orienting to the scents. During and just after mating, males deposit mandibular gland scent on females which is believed to render them temporarily unattractive to other males. Vinson *et al.* (1981) described the mandibular gland and identified the chemical components of the included scent of *C. adani*.

It is likely that glandular scents play an important role in territorial and mating behavior in other *Centris* species. We describe in this paper the mandibular glands and chemical analyses of the glandular contents of eight *Centris* species in three different subgenera from the Costa Rican dry forest. We also provide a brief description of the territorial behavior in two of these species.

MATERIALS AND METHODS

Centris males listed in Table 1 were collected in March 1980 from flowering crowns of several tree species in the lowland dry forest of Guanacaste Province, Costa Rica (see Frankie *et al.*, 1983 for tree list). Males were netted, anesthetized with CO_2, identified by comparison to voucher specimens, and sorted by species into glass vials. Vials were then placed in an ice chest and transported to the field laboratory.

At the field laboratory, mandibles and attached glands were removed from four males of each species for illustration purposes. Drawings were made with the aid of a dissecting microscope that was equipped with a calibrated reticle. Mandibles and attached glands of remaining bees were pooled by species and stored in vials containing glass-distilled hexane. Samples were held below 5°C at the field laboratory until they were transported at ambient temperatures to College Station where they were held at −20°C until their analysis (within 10 weeks).

Mandibular glands were macerated and the resulting extracts analyzed. Gas chromatographic analysis was performed using a Tracor MT-550 GC with flame ionization detection. A 1.83 m × 4 mm i.d. silanized column packed with 3% OV 101 on chromosorb G 80-100 mesh, AW, HMDS, with a nitrogen flow rate of 60 ml/min, programmed from 80 to 250°C at 10°/min, was used to analyze volatile components. Since further separation of several components (eluting at 150°C) was necessary, a second 1.83 m × 4 mm i.d. silanized glass column packed with 5% Carbowax 20 M on chromosorb G, 80–100 mesh, AW, HMDS was used with temperature programmed from 140 to 200°C at 10°/min.

Mass spectral data were obtained on a Varian MAT CH-7 90° sector magnetic scan GC-MS unit equipped with a Varian 620 L data system. The column used was a 1.83 m × 0.2 mm, 3% CW 20 M on chromosorb G, 80/100 mesh, with a helium flow rate of 30 ml/min and temperature programmed from 80 to 250°C at 10°/min. Molecular weights of the unknown compounds were determined from mass spectral data and possible molecular formulas were computed. Analysis of MS breakdown products and data comparison with mass spectra of standards allowed identification of compounds in Table 1. Gas chromatographic analysis by co-injection of commercial samples of these compounds aided the identification (Vinson *et al.*, 1981).

RESULTS AND DISCUSSION

Drawings of the mandible and associated gland from males of seven *Centris* species are provided in Fig. 1. The greatly enlarged mandibular glands of *C.*

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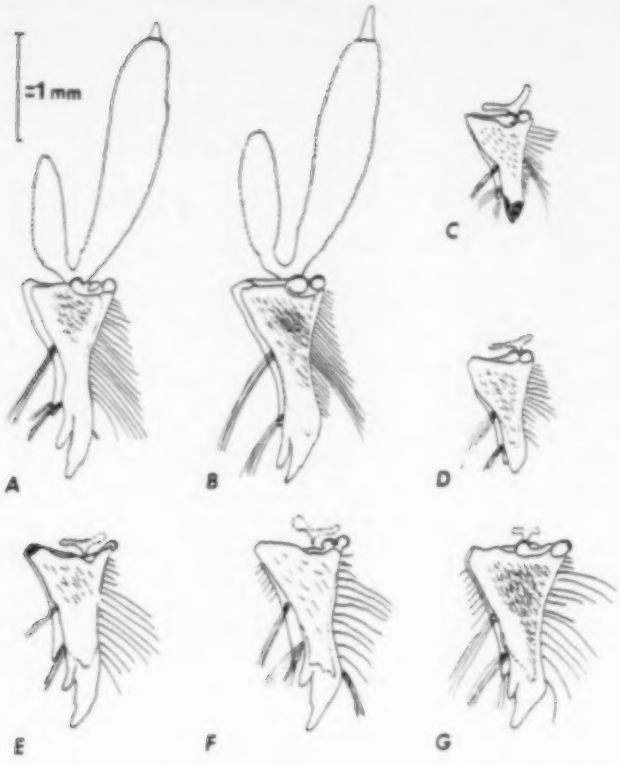

Fig. 1. The mandible and associated gland of male *Centris* species collected from the dry forest of Costa Rica. A = *C. segregata*, B = *C. inermis*, C = *C. nitida*, D = *C. trigonoides subtarsata*, E = *C. aethyctera*, F = *C. heithausi*, and G = *C. fuscata*.

inermis and *C. segregata* (subgenus *Centris*) occupied much of head capsule region. Further, males of these species have been observed maintaining and scent marking territories. This behavior consisted largely of patrolling a limited air space (<1 m^3) adjacent to certain small trees and occasionally alighting on and scent marking twiglets of the trees. The residual odor, which had a citronella fragrance, was easily detected by humans (Frankie, Coville and Vinson, unpublished observations). Similar mandibular gland sizes and spatial arrangements in the head capsule were recorded for *C. adani* (Vinson *et al.*, 1981) and *C. decolorata* Lepeletier and *C. dirrhoda* Noure (Raw, 1975). In the case of *C. adani*, males marked territories (Frankie *et al.*, 1980) with compounds from enlarged mandibular glands (Vinson *et al.*, 1981). Raw (1975) also reported that *C. decolorata* and *C. dirrhoda* scent marked territories, and he suspected the mandibular glands as the scent source.

Appreciable quantities of volatile chemical constituents were recorded in the mandibular glands of only two species: *C. inermis* and *C. segregata* of the

Table 1. Percentage composition of volatiles isolated from mandibular glands of males of eight *Centris* species

Centris species	Number of males from which glands were pooled	Percentages							
		Neral	Nerol	Geranial	Geraniol	Nerolic acid	Geranic acid	Geranyl acetate	Ethyl laurate
Subgenus *Centris*									
*C. adani**	20	0.4	0.8	—	81.1	—	—	17.2	0.6
C. segregata	23	9.2	2.6	42.9	45.2	T†	T	—	—
C. inermis	29	6.7	1.7	15.3	73.7	—	—	—	—
C. aethyctera	82	—	—	—	—	—	—	—	—
Subgenus *Trachina*									
C. heithausi	35	—	—	—	—	—	—	—	—
C. fuscata	36	—	—	—	—	—	—	—	—
Subgenus *Hemisiella*									
C. nitida‡	42	—	—	—	—	T	T	—	—
C. trigonoides subtarsata‡	26	—	—	—	—	—	—	—	—

*Data from Vinson *et al.*, 1981.
†T = Trace of compound detected.
‡Leg glands contain chemicals used in marking territories. See Williams *et al.*, 1983.

subgenus *Centris* (Table 1). Previously published data for *C. adani* are provided for comparison in Table 1 (see Vinson *et al.*, 1981). The major chemical difference between *C. inermis* and *C. segregata* was in the predominance of geraniol in the former species. Relatively equal and large proportions of geraniol and geranial characterized the gland contents of *C. segregata*. Mandibular glands of *C. adani* contained geraniol as the major component with geranyl acetate as a minor component.

Centris aethyctera (subgenus *Centris*) had poorly developed mandibular glands. We have observed this species commonly setting up and maintaining territories without apparent marking. Raw (1975) noted that another species, *C. fasciata* Smith, of the same subgenus had small mandibular glands and also did not scent mark territories. Subgenus *Centris* therefore contains some species in which males do not scent mark their territories and other species, such as *adani*, *decolorata*, *dirrhoda*, *segregata* and *inermis*, in which males mark territories with mandibular gland scents.

In the other four species (*nitida*, *trigonoides subtarsata*, *fuscata* and *heithausi*) the presence of small mandibular glands (Fig. 1) containing little or no volatiles (Table 1) suggested that mandibular gland scents are unimportant in their mating behavior. However, males of *C. nitida* and *C. trigonoides subtarsata*, subgenus *Hemisiella*, used hind tibial gland scents to mark their territories (Williams *et al.*, 1983; Frankie, Vinson and Coville, unpublished data). Raw (1975) reported that males of *C. crassipes* Smith in the same subgenus probably also use scents from hindleg glands to mark territories.

Centris heithausi and *C. fuscata* are in the subgenus *Trachina* (Snelling, 1974), which according to Michener (1951) is closely related to *Hemisiella*. We have observed both species regularly setting up and maintaining territories; however, scent marking behavior appears to be a rare event. Further, we have found that *C. heithausi* has small or poorly developed tibial glands and compounds similar to those produced by the tibial glands of *C. nitida* and *C. trigonoides subtarsata* have been identified (Williams, Vinson, Frankie and Coville, unpublished data). We have not found tibial glands in any of the species in the subgenus *Centris*.

Studies by Alcock (1976) and Alcock *et al.* (1976, 1977) indicate that males of *Centris pallida* Fox, subgenus *Xerocentris*, do not scent mark territories. Information is lacking for representative species in the numerous other subgenera of *Centris*. Thus, at present there is only a fragmentary picture of the subgeneric distribution of territorial scent marking behavior by *Centris* males.

That *Centris* bees with enlarged mandibular glands utilize different ratios of the same compounds for scent marking is not surprising in view of findings on other species that use multi-component pheromone systems. For example, species of dermestid and *Ips* beetles and leafroller moths are known to use varying ratios of chemical components as attractants (Greenblatt *et al.*, 1977; Birch *et al.*, 1980; Bjostad *et al.*, 1980). A similar pattern is found in *Andrena* bees (Tengö and Bergström, 1976). Species specificity in each genus is obtained by adding or subtracting components or by varying isomeric composition of these components. Greenfield and Kanandions (1979) in discussing the Sesiidae pheromone compositions, state that specific component mix ratios become important in promoting single species encounters when isolating mechanisms such as temporal and spatial segregation are absent. Since many of the sympatric *Centris* species in the Costa Rican dry forest have enlarged mandibular glands and similar mating habits, chemical isolation may be especially important. All *Centris* species that are known to use tibial gland secretions for marking use a completely different set of chemicals, again in varying ratios (Williams *et al.*, 1983). Findings such as these should prove helpful in elucidating chemical/ecological and phylogenetic relationships in this genus.

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ACTIVATION OF RESPIRATION IN SEA URCHIN SPERMATOZOA BY CALCIUM IONOPHORE A23187

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Abstract—1. The respiratory rate in spermatozoa of the sea urchin, *Hemicentrotus pulcherrimus*, in Na^+-free seawater, where sperm are immotile and their respiration remains inactive, was stimulated by calcium ionophore A23187.

2. Addition of ionophore A23187 to Na^+-free seawater induced swimming as well as activating energy metabolism in sea urchin sperm.

3. The increase of respiratory rate and the initiation of motility in sperm were independent of external Ca^{2+}.

INTRODUCTION

Sea urchin spermatozoa are immotile in "dry sperm" and their rate of respiration is extremely low (Mohri and Yasumasu, 1963). Once diluted in sea water, sperm begin to swim and the respiratory rate increases (Rothschild, 1951; Mita and Yasumasu, 1983a). After dilution in Na^+-free seawater, however, sperm are immotile and the respiration remains inactive (Morisawa, 1958; Mita and Yasumasu, 1983b). The energy metabolism in sperm diluted in Na^+-free sea water is also inactive (Mita and Yasumasu, 1983b). Therefore, external Na^+ plays an important role for initiation of sperm movement and activation of respiration, resulting from that the Na^+-dependent acid release (Nishioka and Cross, 1978) and the increase of internal pH (Lee *et al.*, 1983) follow dilution.

On the other hand, Ca^{2+} has been implicated as a second messenger in cellular responses to their action. In sea urchin sperm, it is well known that Ca^{2+} is required for the acrosome reaction (Dan, 1954). The swimming rate of sea urchin sperm is also depressed by EDTA† and EGTA (Young and Nelson, 1974). Hence, it is supposed that Ca^{2+} plays a role in the activation of sperm.

This report studies the effect of calcium ionophore A23187 on the respiration and the movement in sea urchin sperm. The results obtained suggest that the increase of the intracellular free Ca^{2+} concentration triggers the activation of respiration and the initiation of motility in sperm upon dilution in seawater.

MATERIALS AND METHODS

Materials

Spermatozoa of the sea urchin, *Hemicentrotus pulcherrimus*, were obtained by forced spawning induced by 0.5 M KCl injection into the coelomic cavity. Semen was collected as "dry sperm" and kept undiluted at 4°C. Artificial seawater (ASW) contained 485 mM NaCl, 9.6 mM KCl, 10 mM $CaCl_2$, 49 mM $MgSO_4$ and 10 mM Tris–HCl, pH 8.0. In Na^+-free seawater (KSW), KCl was substituted for NaCl. Ca^{2+}-free seawater (CAFSW) contained 473 mM NaCl, 9.6 mM KCl, 49 mM $MgSO_4$ and 10 mM Tris–HCl, pH 8.0. Calcium ionophore A23187 was purchased from CalBiochem Co., USA. Calcium ionophore A23187 dissolved in dimethylsulfoxide (DMSO) was used for the experiments.

Oxygen consumption

Sperm respiration was estimated polarographically on a Clark-type oxygen electrode (Yellow-Springs Co., USA). A 100-fold dilution of dry sperm was made into 2.5 ml of seawater at 20°C.

Motility

Estimates of motility were obtained by light microscopic observation on a thick droplet of sperm suspension. Sperm motility was scored qualitatively in 5 categories as follows: almost all sperm are briskly moving (+++), almost all sperm are moving (++), almost 50% of sperm are moving (+), few sperm are still moving (±) and all sperm are immotile (−).

ATP assay

The specimens for determination of ATP were incubated for the desired time, after 100-fold dilution of the dry sperm in seawater at 20°C. After centrifugation of each sample at 3000 *g* for 3 min at 0°C, the pellet was mixed with 4 volumes of 0.8 M perchloric acid before being homogenized with a teflon homogenizer. The acidified homogenate was centrifuged at 10,000 *g* for 10 min at 4°C. The supernatant was used for the estimation of ATP after neutralization to pH 6.5 with KOH. ATP concentration was determined enzymatically as described (Lamprecht and Transchild, 1974).

Protein determination

Protein was measured by the method of Lowry *et al.*

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†Abbreviations used: ASW, artificial seawater; KSW, Na^+-free seawater; CAFSW, Ca^{2+}-free seawater; EDTA, ethylenediamine tetraacetic acid; EGTA, ethyleneglycol-bis(β-amino-ethyl ether)*N*,*N*′-tetraacetic acid; DMSO, dimethylsulfoxide.

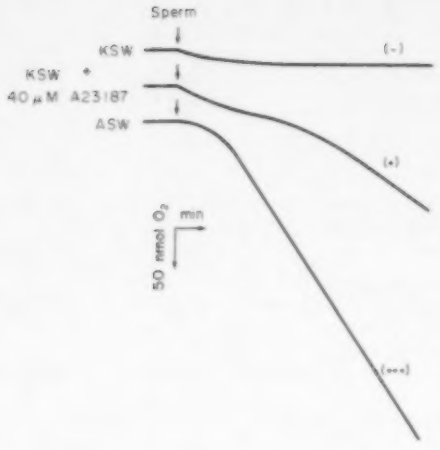

Fig. 1. Trace of change in the oxygen concentration in suspension of sea urchin spermatozoa. The "dry sperm" were diluted 100 times in artificial seawater at 20°C. Arrows show the times of sperm addition. Parentheses represent the motility category as described in "Materials and Methods".

(1951) with bovine serum albumin as standard. Approximately 2×10^9 sperm corresponded to 1 mg protein.

RESULTS

When dry sperm of sea urchin were diluted in the ASW, sperm began to swim and their respiratory rate was activated as in the previous study (Mita and Yasumaus, 1983a). However, sperm diluted in Na^+-free seawater (KSW) remained immotile and the oxygen consumption was extremely low. Addition of calcium ionophore A23187 to the KSW stimulated motility and respiration (Fig. 1). DMSO (maximal concentration 1%) which is a solvent for the ionophore had no effect on the sperm movement and respiration. A sigmoid curve was observed in the trace of the oxygen consumption stimulated by the ionophore and 2–3 min after addition of the ionophore the rate of respiration reached a constant level (Fig. 1). The respiratory rate and the swimming speed after addition of the ionophore were, however, inferior to those after dilution in the ASW. In the case of sperm diluted in the ASW, their motility and respiration were not influenced by adding of calcium ionophore A23187 (data not shown).

The increase of rate of respiration depended upon the concentration of calcium ionophore A23187 (Fig. 2). The respiratory rate in the presence of 5 μM ionophore was 5 times higher than that in the absence of the ionophore. Where 100 μM ionophore A23187 was added to the KSW, the respiratory rate increased 10 times against without the ionophore but the stimulated oxygen consumption was only 60% of that obtained in dilution in the ASW.

It is known that the level of ATP in sea urchin sperm decreases after dilution in seawater (Taguchi *et al.*, 1963; Yanagisawa, 1967; Mita and Yasumasu, 1983a). This indicates that ATP is consumed by dynein ATPase concomitantly with the initiation of movement in sea urchin sperm. After dilution in a Na^+-free seawater, such as 0.5 M KCl or 0.75 M sucrose, the ATP level hardly changes (Morisawa, 1958; Mita and Yasumasu, 1983b). On the other hand, where calcium ionophore A23187 was added to the KSW, the ATP level decreased after dilution in the KSW (Fig. 3). The change in the ATP level after dilution in the KSW containing the ionophore showed a similar result to that in dilution in ASW.

Furthermore, when sperm were diluted in Ca^{2+}-free seawater (CAFSW), they were able to swim and the respiration was activated (Table 1). There was no difference between dilution in the ASW and the CAFSW on the respiratory rate and swimming activity. However, sperm were immotile and their respiration was low after dilution in KSW which contained 10 mM $CaCl_2$ (Fig. 1 and Table 1). These results suggest that the external Ca^{2+} is not related directly to the initiation of motility and the activation of respiration upon dilution in seawater.

DISCUSSION

The Na^+-dependent acid release occurs concomitantly with the initiation of sperm motility (Nishioka and Cross, 1978). The internal pH in sea urchin sperm increases accompanied with the exchange of Na^+/H^+ (Lee *et al.*, 1983). In the Na^+-free seawater, sperm can begin to swim on addition of NH_4^+ or at pH 9 (Nishioka and Cross, 1978; Lee *et al.*, 1983). It is also possible that the increase of internal pH following dilution in seawater induces activation of the mitochondrial function. This is related to that the "burst" in respiration following dilution, the so called "dilution effect" (Mohri and Yasumasu, 1963).

The activation of mitochondria is associated with the internal Ca^{2+} concentration. The respiration in sea urchin sperm was stimulated by the addition of calcium ionophore A23187 to the KSW (Fig. 1). This result suggests that the increase of respiratory rate

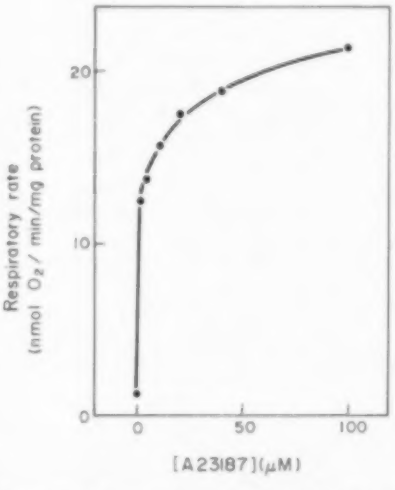

Fig. 2. Effect of calcium ionophore A23187 on the rate of respiration in sea urchin spermatozoa. The "dry sperm" were diluted 100 times in the Na^+-free seawater containing the indicated concentration of calcium ionophore A23187 at 20°C. Control experiment was carried out in the presence of 1% DMSO.

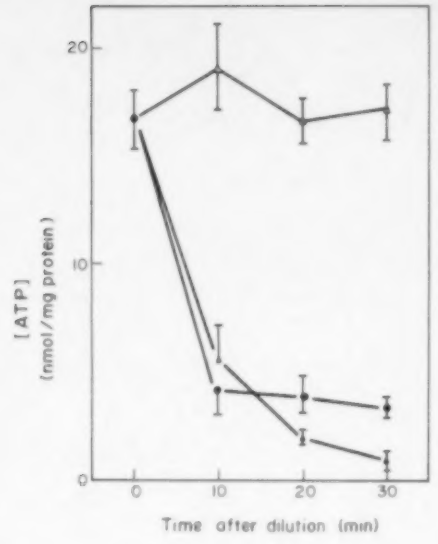

Fig. 3. Change in the level of ATP in sea urchin spermatozoa after dilution in artificial seawater. The "dry sperm" were diluted in 100 times in ASW (●), KSW (△) and KSW containing 20 μM calcium ionophore A23187 (▲) at 20°C. Each mark represents the average value obtained from three separate experiments and bars show the standard error of mean.

after dilution in seawater is caused by the increase of internal free Ca^{2+} concentration. The level of ATP in sea urchin sperm represents the apparent amount obtained from difference between the consumption for the movement and the production for the oxidative phosphorylation in mitochondria. The change in ATP level after dilution in the KSW containing the ionophore showed the same response as that after dilution in the ASW (Fig. 3). These suggest that the stimulated respiration by the ionophore is closely correlated to the motility. It seem however, that the swimming activity and the respiratory rate induced by the ionophore are inferior to those in dilution in the ASW (Fig. 2). It is probable that dynein ATPase and the mitochondrial enzymes do not exert a maximal activity in the intracellular condition of low pH. Without external Na^+, sperm may not be able to carry out the alkalinization of the KSW.

Ca^{2+} plays a important role for the acrosome reaction in sea urchin sperm (Dan, 1954). The acrosome reaction is also induced by calcium ionophore A23187 (Summers *et al.*, 1976; Talbot *et al.*, 1976). However, at the condition of pH 8 used in the present study, the ionophore hardly induced the acrosome reaction (Decker *et al.*, 1976). The rate of respiration and the activity of swimming in sperm were not influenced by the addition of calcium ionophore A23187 to the ASW (data not shown). These suggest that the stimulated respiration and movement by the ionophore is distinguished from a second effect in succession to the acrosome reaction. The external Ca^{2+} is required for the acrosome reaction (Dan, 1954) but not for the activation of respiration and movement following dilution (Table 1). This agrees with the report (Schackmann *et al.*, 1978) that there is no Ca^{2+} uptake in the activation of sperm following dilution. The present study also shows that sperm remained immotile and their respiratory rate was low after dilution in the KSW containing 10 mM $CaCl_2$ (Table 1), suggesting that the sperm activation following dilution is independent of external Ca^{2+}.

Table 1. Effect of Ca^{2+} on respiratory rate of sea urchin spermatozoa

Condition	Respiratory rate (nmol O_2/min/mg protein)	Motility
ASW	35.0 ± 3.0	+++
KSW	2.6 ± 0.3	−
CAFSW	34.7 ± 2.4	+++

The "dry sperm" were diluted 100 times in artificial seawater (ASW, KSW or CAFSW). The value is mean ± SEM obtained in 3 separate experiments.

It is supposed that there may be three steps for activation of sperm following dilution. CO_2 is a reasonable candidate to inhibit the sperm function in the "dry sperm" (Mohri and Yasumasu, 1963). CO_2 exerts a narcotic effect and depresses the metabolism due to its influence on intracellular pH, resulting from the rapid penetration of CO_2 across the cell membrane. Once "dry sperm" are diluted in seawater, the CO_2 effect is cancelled easily. The exchange between Na^+ and H^+ occurs upon dilution and the internal pH increases concomitantly with the acid release (Nishioka and Cross, 1978; Lee *et al.*, 1983). Then free Ca^{2+} may be released from an intracellular store following the alkalinization. Movement and respiration may be activated by the increase of internal Ca^{2+} concentration and the alkalinization. On the other hand, it has been demonstrated that the Na^+-dependent acid release and the increase of internal Ca^{2+} concentration happen in sea urchin eggs upon fertilization (Johnson *et al.*, 1976; Steinhardt *et al.*, 1977). Sea urchin eggs can be activated by calcium ionophore A23187 in Ca^{2+}-free seawater (Chambers *et al.*, 1974; Steinhardt and Epel, 1974). It is interesting that the mechanism of activation in sperm following dilution in sea water may be similar to that of activation in eggs following fertilization.

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PATTERNS OF ACID–BASE REGULATION IN URODELE AMPHIBIANS IN RESPONSE TO VARIATIONS IN ENVIRONMENTAL TEMPERATURE

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Abstract—1. Larval and adult *Ambystoma tigrinum*, adult *Amphiuma means* and *Necturus maculosus* were acclimated to 5, 15 and 25°C for analysis of blood pH, P_{CO_2}, and $[HCO_3^-]$.

2. They demonstrated the typical poikilothermic maintenance of "relative alkalinity" by significantly decreasing their pH with warming; $\delta pH/\delta T$ ranged from −0.015 to −0.018.

3. *Necturus* remained a water breather with constant P_{CO_2} (4.8–5.6 torr) and significantly decreased $[HCO_3^-]$ (19.5–8.6 mM) with warming.

4. Larval *A. tigrinum* and *Amphiuma*, water breathing at 5°C and then water–air breathing at 15 and 25°C, significantly increased P_{CO_2} (3.9–13.6 torr and 5.8–12.5 torr, respectively) with warming. Larval *A. tigrinum* significantly increased their $[HCO_3^-]$ (17.5–26.9 mM) while *Amphiuma* made insignificant change (30.4–22.4 mM).

5. Adult *A. tigrinum* remained an air breather with a relatively elevated P_{CO_2} which significantly rose (9.1–14.2 torr) and an $[HCO_3^-]$ which significantly decreased (35.8–22.2 mM) with warming.

6. This suggests that these urodeles manipulate their extracellular P_{CO_2} and $[HCO_3^-]$ relationship as they pursue maintenance of "relative alkalinity" during thermal acclimation and altered metabolism.

INTRODUCTION

The invasion of terrestrial habitats by animals originally adapted for aquatic respiration facilitated their acquisition of oxygen. However, a price was paid in impaired CO_2 elimination (Rahn, 1966). It has been suggested that this led to a potential respiratory acidosis which was compensated for by the accumulation of bicarbonate ion (Howell, 1970). Much of the evidence in support of this proposition has come from data taken from fish (Randall and Cameron, 1973) and anuran amphibians (Howell *et al.*, 1970; Erasmus *et al.*, 1970; Just *et al.*, 1973).

The group of amphibians which are believed to be most closely related to the vertebrate animals which first made the transition to aerial respiration belong to the order Urodela. Animals in this group utilize a wide variety of respiratory strategies ranging from pure water breathers to pure air breathers with various intermediate patterns of water–air respiration interposed. In addition, switching from one breathing mode to another can be observed not only between larval and adult forms of the same species but also within the same morph during temperature acclimation. This diversity within a closely related group provides an excellent opportunity to test both the bicarbonate retention hypothesis of Howell (1970), and the "relative alkalinity" hypothesis proposed by Rahn (1967) for poikilotherms during temperature acclimation.

Very little is known about acid–base balance in urodeles. Moalli *et al.* (1981) have published a rather complete picture of acid–base adjustments to temperature for *Cryptobranchus alleganiensis* which is a water-breathing salamander. A limited amount of data for water–air breathing larval and air-breathing adult *Ambystoma tigrinum* are available (Burggren and Wood, 1981). These latter data, however, do not entirely confirm the predictions of Howell (1970). Despite the fact that their larvae increased aerial respiration upon warming from 5 to 25°C, there was a negligible increase in P_{CO_2} and a 30% reduction in bicarbonate concentration.

Our objective in this study was to evaluate the effects of temperature on acid–base balance in three species of salamanders which exhibit different patterns of respiration. *Necturus maculosus* is a pure water-breather between 5 and 25°C (Guimond and Hutchinson, 1972). *Amphiuma means* is a gill-less cutaneous water-breather at 5°C but becomes a pulmo-cutaneous air–water breather at 25°C (Guimond and Hutchison, 1974). Larval *Ambystoma tigrinum* utilize pulmo-cutaneous gill air–water breathing, while adults of this species are pulmo-cutaneous air-breathers (Whitford and Sherman, 1968).

MATERIALS AND METHODS

Animal maintenance

Larval *Ambystoma tigrinum* weighing between 15 and 45 g were obtained from a local bait dealer. Neotenic *Necturus maculosus* (170–190 g), adult *Amphiuma means* (250–400 g) and adult *A. tigrinum* (15–25 g) were obtained from Rand–McNalley Biocenter (Somerset, WI). A few adult *A. tigrinum* were metamorphosed in the laboratory by introducing *ca* 1 mg/l triidothyronine into the bathing water. Aquatic salamanders were maintained at 5°C in an Instant Ocean aquarium adapted for fresh water use. Adult

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A. tigrinum were maintained on moist paper toweling at room temperature.

Thermal acclimation

Groups of adult *A. tigrinum* were placed in a plastic box with tap water access and covered with an insulated (styrofoam) lid. Humidified air was circulated through the box which was partially submerged in a constant temperature bath. Each group was then acclimated to either 5, 15 or 25°C (±1°C) for 3 days. Separate groups of larval *A. tigrinum* and *Necturus* were placed in aerated tap water and acclimated to the same temperatures for 3 days. Individual *Amphiuma* were acclimated to 5 and 25°C in aerated tap water for 3 days.

Blood collection

The small size of *A. tigrinum* made the implantation of chronic indwelling catheters impractical so we resorted to a modification of a technique used previously on bullfrog tadpoles and small adults (Just *et al.*, 1973). Larvae and adults were gently removed from their acclimation chambers, quickly decapitated, spinally pithed and opened to expose the heart and large vessels within 10 sec. Blood was collected with heparinized collecting tubes following insertion in the severed conus arteriosus. The blood filled the tube by the pumping action of the heart and samples which became contaminated with air bubbles were not included in the study. Animals which struggled prior to pithing or which required more than one minute to complete blood sampling were excluded from the study. In order to insure that this technique adequately prevented the production of lactic acid and subsequent artifaction of the acid–base balance, we measured pH and lactic acid concentration (Sigma Chemical Company lactic acid dehydrogenase method) on a separate group of larval *A. tigrinum* adapted to 25°C. We were able to obtain data from animals which met the same criteria for absence of struggling described above as well as data from animals which struggled. The results from seven larvae which did not struggle were a mean pH of 7.78 (±0.03 SEM) and lactic acid concentration of 0.67 mM (±0.09 SEM). The lactic acid concentration is below that reported by Hutchison *et al.* (1977) for resting, unrestrained *A. tigrinum* and is not significantly different from the lactic acid concentration found in the catheterized resting *Cryptobranchus* (Boutilier *et al.*, 1980). The pH values are not significantly different from the rest of the larvae sampled at 25°C. In contrast, the four larvae which struggled and would therefore have been excluded from the study had a significantly lower mean pH of 7.56 (±0.11 SEM) and higher lactic acid concentration of 1.06 mM (±0.11 SEM). The pH values which were obtained from acceptable sampling procedures are in the same range as those reported for other amphibian species (Howell *et al.*, 1970). The same sampling procedure was employed for *Necturus*. The much larger *Amphiuma* were equipped with in-dwelling arterial catheters. The animals were anesthetized in 0.15‰ tricaine methanesulfonate (Cresent Biochemicals, Paradise Valley, AZ), buffered to pH 7.0 with Na HCO_3. Animals were then draped with wet toweling, incised to expose the viscera and cannulated with PE 50 tubing via the coeliaco-mesenteric artery. The cannula was filled with amphibian Ringer's solution containing 35 IU heparin/ml. The animals were sampled during free swimming periods 24 hr after surgery following the third day of temperature acclimation (5 or 25°C).

Measurement of acid–base parameters

Blood pH was measured at the acclimation temperature using an Instrumentation Laboratories blood gas analyzer (model 113) and temperature corrected buffers. Blood P_{CO_2} was measured by the Astrup technique (1956) at the acclimation temperature by tonometering blood with 4 and 8% CO_2. This method assumes that the log P_{CO_2}–pH relationship is linear over a wide-range of P_{CO_2}. Astrup (1956) reported that between 12 torr and 75 torr, the relationship is quite linear in mammalian blood. Wood *et al.* (1982) found a linear relationship for fish blood down to 5 torr. Our P_{CO_2} measurements ranged from approximately 4–14 torr for the four urodele types studied. The potential for error in these measurements affects primarily the water breathing forms whose P_{CO_2} is approximately 5 torr. The nonlinearity reported by Wood *et al.* (1982) in the 1–5 torr range might cause our estimates of P_{CO_2} in this range to slightly overestimate the true P_{CO_2} by 1–2 torr. Thus, the differences we report between water-breathers and air-breathers for P_{CO_2} may actually be greater than our data show.

Bicarbonate concentration was determined by using the Henderson–Hasselbalch equation. The pK' and alpha CO_2 values were temperature corrected according to Reeves (1976). Because of the nonlinearity of the log P_{CO_2}–pH relationship in the 1–5 torr range, the bicarbonate concentration for water-breathing forms also should be considered an upper limit value.

Statistics

Significance of observed differences were assessed by using analysis of variance, Newman–Keuls multiple range, and Student's *t*-test for unpaired samples. Where differences are stated to be significant, the level of probabilities were found to be 0.05 or less.

RESULTS

The respiratory patterns of the different salamanders differed substantially over the temperature range studied. *Necturus* remained submerged at all temperatures and relied entirely on gill–skin aquatic respiration. *Amphiuma* remained submerged and employed aquatic skin respiration at 5°C and began to surface 2–5 times/hr to engage in aerial pulmonary and aquatic skin respiration at 25°C. Larval *A. tigrinum* displayed gill–skin aquatic respiration at 5°C. At 15°C, they began to surface 3–6 times/hr and then 5–10 times/hr at 25°C for aerial pulmonary and gill–skin aquatic respiration. Adult *A. tigrinum* utilized cutaneous–pulmonary aerial respiration at all temperatures.

All of the species decreased blood pH as temperature increased from 5 to 25°C (Table 1 and Fig. 1). In each case, the pH decreased significantly as the temperature was increased to the next level. The patterns of decreasing pH with increasing temperature were consistent in that each species maintained a "relative alkalinity" over the observed temperature range. The temperature coefficient ($\delta pH/\delta T$) for the groups was: adult *A. tigrinum*, −0.016; larval *A. tigrinum*, −0.015; *Amphiuma*, −0.018; and *Necturus*, −0.016.

The differences in respiratory patterns displayed parallel changes in P_{CO_2} and bicarbonate concentration profiles (Figs 2 and 3). *Necturus* maintained a constant P_{CO_2} of approximately 5 torr over the entire range of temperature acclimation. Bicarbonate concentration significantly decreased with temperature. *Amphiuma* maintained a low P_{CO_2} of 5.8 torr during cutaneous aquatic respiration at 5°C; this increased significantly to 12.5 torr with the addition of aerial respiration at 25°C. Bicarbonate concentration did not change significantly with warming. Larval *A. tigrinum* increased P_{CO_2} significantly at each

Table 1. Respiratory behavior and blood pH of urodele amphibians adapted to different ambient temperatures

Species	Temperature (°C)	Breathing mode	pH
Ambystoma tigrinum adult	5	Air	8.06 ± 0.04 (9)
	15	Air	7.92 ± 0.01 (9)A
	25	Air	7.75 ± 0.01 (7)B
Ambystoma tigrinum larvae	5	Water	8.11 ± 0.02 (16)
	15	Water–air	7.94 ± 0.02 (15)A
	25	Water–air	7.81 ± 0.02 (22)B
Amphiuma means	5	Water	8.15 ± 0.03 (6)
	15	Water–air	7.79 ± 0.06 (6)A
Necturus maculosus	5	Water	8.05 ± 0.02 (10)
	15	Water	7.90 ± 0.01 (9)A
	25	Water	7.74 ± 0.02 (10)B

Values are means ± SEM and the number of measurements in parentheses.
A = significantly different ($P < 0.05$) to 5°C data. B = significantly different ($P < 0.05$) to both 5 and 15°C data.

step increase in temperature. Their P_{CO_2} of 3.9 torr at 5°C while water-breathing is not significantly different from either *Amphiuma* or *Necturus* at the same temperature. Bicarbonate concentration increased significantly during the transition from 5 to 15°C, and from 15 to 25°C. Adult *A. tigrinum* maintained elevated P_{CO_2} of 14.2 torr at 25°C, which is not significantly different from the air–water breathing *Amphiuma* and larval form at the same temperature. At 5°C, they maintained a greater P_{CO_2} (9.1 torr) than any of the other water-breathing salamanders. Bicarbonate concentration decreased significantly between 5 and 15°C, but insignificantly between 15 and 25°C. In addition, their bicarbonate concentrations remained higher than those maintained by the pure-water breathing forms at all temperatures.

DISCUSSION

Each of the four forms of salamanders displayed the pattern of decreasing blood pH with the increasing temperature consistent with the maintenance of "relative alkalinity" which is characteristic of poikilothermic animals as predicted by Rahn (1966) and demonstrated by Howell *et al.* (1970). The values we found for $\delta pH/\delta T$ are quite similar to those generally observed for poikilotherms (Reeves, 1977). Our data for larval and adult *A. tigrinum* are in

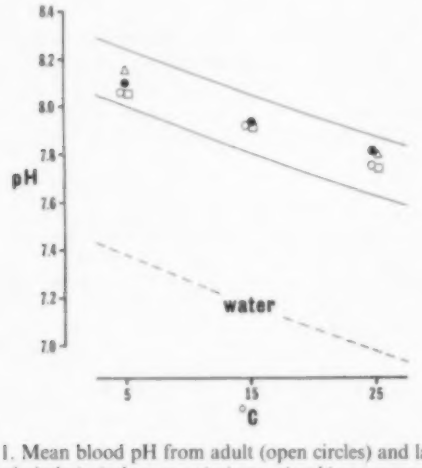

Fig. 1. Mean blood pH from adult (open circles) and larval (closed circles) *Ambystoma tigrinum*, *Amphiuma means* (triangles), and *Necturus maculosus* (squares) as a function of body temperature. The bracketing band is parallel to the neutral pH of water and represents the range of reported poikilothermic blood pH values.

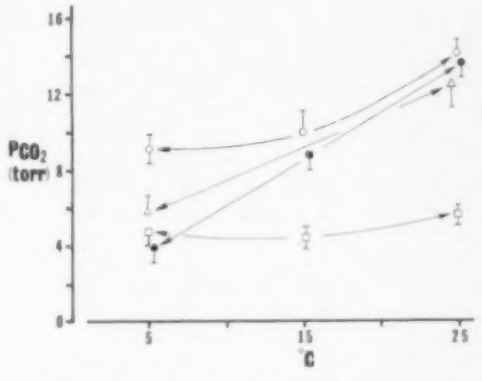

Fig. 2. Mean blood P_{CO_2} (±SEM) from adult (open circles, $N = 8$/group) and larval (closed circles, $N = 13$/group) *Ambystoma tigrinum*, *Amphiuma means* (triangles, $N = 6$/group), and *Necturus maculosus* (squares, $N = 9$/group) as a function of body temperature.

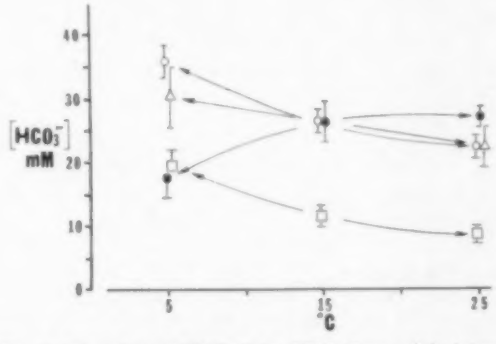

Fig. 3. Mean blood $[HCO_3^-]$ (±SEM) from adult (open circles, $N = 8$/group) and larval (closed circles, $N = 13$/group) *Ambystoma tigrinum*, *Amphiuma means* (triangles, $N = 6$/group), and *Necturus maculosus* (squares, $N = 9$/group) as a function of body temperature.

general agreement with those of Burggren and Wood (1981) who reported a $\delta pH/\delta T$ approximately -0.014 between 5 and 25°C. Another urodele, *Cryptobranchus alleganiensis*, has a reported temperature coefficient of -0.016 (Moalli *et al.*, 1981).

The patterns of P_{CO_2} and bicarbonate concentration vs temperature differ greatly among the four forms studied. These differences are, in general, consistent with differences in respiratory strategies displayed by these salamanders. *Necturus*, remaining a water-breather, is seen to maintain the predicted upper limit of P_{CO_2} (5 torr) throughout the 5 to 25°C temperature range in accordance with the aquatic gas exchange theory (Rahn, 1966). This, despite a five-fold increase in CO_2 excretion by its gill–skin respiratory mode (Guimond and Hutchison, 1972). *Necturus* pursues "relative alkalinity" by decreasing its bicarbonate concentration 56% as temperature increased. This pattern is very similar to that displayed by the teleost fish also constrained by aquatic gas exchange as it maintains "relative alkalinity" (Randal and Cameron, 1973). Moalli *et al.* (1981) have reported that the water-breathing *Cryoptobranchus* increases P_{CO_2} with temperature without resorting to air-breathing as it maintains "relative alkalinity". The difference between *Cryoptobranchus* and *Necturus* may be related to the latter having an extensive gill surface with which to diffuse the increasing CO_2 load as both are warmed. This increase in P_{CO_2} apparently allows *Cryoptobranchus* to maintain "relative alkalinity" without significantly adjusting its bicarbonate concentration, while *Necturus* must because of its lower P_{CO_2} maintenance. The passive diffusion-limited CO_2 exchange apparent in *Cryoptobranchus* suggests little control over CO_2 exchange when compared to *Necturus* who maintains a fairly constant P_{CO_2} despite a similar increase in CO_2 production with increasing temperature.

Adult *A. tigrinum* are air-breathers at all temperatures observed in this study. In accordance with Rahn's predictions (1966), they maintained blood P_{CO_2} levels higher than their water-breathing counterparts at each temperature. They do, however, tend to have higher P_{CO_2} at higher temperature like *R. catesbieana* (Howell *et al.*, 1970). Burggren and Wood (1981) also observed an increase in P_{CO_2} between 5 and 25°C in adult *A. tigrinum*. Consistent with their relatively elevated blood P_{CO_2} levels, they displace their blood bicarbonate to higher levels than found in the water-breathing *Necturus* and the low temperature (5°C) water-breating larvae of the same species. This, too, is consistent with the predictions of Howell (1970).

Larval *A. tigrinum* maintains a low P_{CO_2} (3.9 torr) at 5°C while in the water-breathing mode by gill–skin gas exchange which is again in accord with aquatic gas exchange theory. Their bicarbonate concentration is 50% less than the adult form which is air-breathing at this temperature. As they are warmed and begin to engage in air-breathing via cutaneous–pulmonary gas exchange, they increase their bicarbonate concentration 54% to enter the same range the adults maintain. Burggren and Wood (1981) do not show *A. tigrinum* larvae to increase their bicarbonate concentration despite their air-breathing behavior and increased CO_2 production. Our data indicates that larval *A. tigrinum* conform to the bicarbonate retention predictions of Howell (1970) as they begin to increase their metabolic rate, air-breathe and subsequently sustain an elevated P_{CO_2}. This same pattern has been observed in metamorphosing anurans (Just *et al.*, 1973).

Amphiuma, displaying a similar water to water/air-breathing behavior, has a curious pattern which does not correspond entirely to any of the other forms. *Amphiuma* exchanges 100% of its CO_2 production via the cutaneous–aquatic route at 5°C and upon warming to 25°C, it begins to utilize the cutaneous–pulmonary aerial mode to excrete 23% (Guimond and Hutchison, 1974). At 5°C, they maintain a P_{CO_2} (5.8 torr) at the upper limit predicted by aquatic gas-exchange theory which is very similar to that seen in *Necturus* and *Cryoptobranchus* (Moalli *et al.*, 1981). As they are warmed, they increase their P_{CO_2} (12.5) suggesting increasing P_{CO_2} as a result of increased CO_2 production and aerial gas-exchange. Bicarbonate concentration was high at both temperatures and the 5°C value is significantly higher than the corresponding value of both of the other aquatic salamanders at the same temperature. This probably reflects the fact that this species frequents hypercapnic environments and maintains a large extracellular bicarbonate store which can be shifted to the intracellular compartment during times of hypercapnic stress (Heisler *et al.*, 1982).

In conclusion, our results demonstrate that these urodeles pursue the maintenance of an extracellular "relative alkalinity" despite wide variations in respiratory behavior, structure and function. They increase P_{CO_2} with the commencement of air-breathing and as the metabolic rate scales with temperature. They generally manipulate bicarbonate concentration in the extracellular space (and presumably the intracellular space) during water, water to air and air-breathing as they maintain "relative alkalinity". The mechanism these animals employ to manipulate their bicarbonate concentration is not well understood. Species of both anurans and urodeles are thought to exchange ions (H^+ for Na^+ and/or Cl^- for HCO_3^-) across skin, gill and renal epithelium which would influence bicarbonate concentration (Alvarado, 1979). The coupling of acid–base, gas and ion exchange needs to be studied at rest and during stress (hypercapnia and/or exercise) in order to clarify the way these animals maintain their "relative alkalinity".

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PHYSIOLOGICAL ADAPTATION OF COTTOID FISHES OF LAKE BAIKAL TO ABYSSAL DEPTHS

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Abstract—1. Pressure tolerance of ten species of shallow water (< 100 m) Cottidae and deep water (300–1600 m) Abyssocottidae, cottoid fishes endemic to Lake Baikal, were explored by measuring threshold pressures for high pressure convulsions.

2. The observed family differences reflect not merely acclimation to habitat pressure, but rather suggest a significant degree of genetic determinacy of this characteristic, pointing to a possible role of hydrostatic pressure as a selection factor in the evolution of deep water faunas.

INTRODUCTION

In contrast to the great volume of work on temperature acclimation and adaptation (cf. e.g. Cossins and Prosser, 1978; Lagerspetz, 1974; Reeves, 1977; Rahman *et al.*, 1980), little has been reported to date documenting the extent to which pressure, the second major extensive thermodynamic parameter characterizing environments, may be a factor of evolutionary significance. In vertebrates, such evidence is almost wholly indirect, based on failure of deep water fish species to survive at 1 ata (Childress *et al.*, 1980), and to suggestive biochemical evidence (Siebenaller and Somero, 1978; Hochachka, 1976). A recent collaborative expedition to Lake Baikal, in central Siberia, afforded an opportunity to carry out a comparative study on a number of related species of swimbladderless (Berra, 1981) cottoid fishes which at least since early Pleistocene times have differentiated there into the shallow water family Cottidae and the deep water family Abyssocottidae (Taliev, 1955; Sidelyova, 1982), each of which in turn has produced a few aberrant species inhabiting depths normally characteristic only of the other family. These "crossover" species promised a unique opportunity to explore the relative importance of genetic as compared to acclimational factors in determining physiologic fitness for life in deep water.

We used the sequence of behavior changes observed in all vertebrates subjected to progressively increasing pressures (Brauer *et al.*, 1981) to assess pressure tolerances of various species of Baikalian cottoids and to explore their possible relation to habitat hydrostatic pressures.

MATERIALS AND METHODS

Fishes were captured by trawling during June and early July when surface temperatures in Lake Baikal exceed bottom temperatures by no more than 3°C (Kozhov, 1962), and placed in holding pans kept at temperatures as near as possible to habitat temperature. Trawling depths were determined by a calibrated precision fathometer. Because these are benthic or benthopelagic species, and because of trawl structure, one can safely conclude that capture depths were equal to trawling depths.

Fish were compressed hydraulically in refrigerated lake surface water at a rate of 500 ata/hr. All experiments were completed within 40 min. Fish were compressed singly, and no fish was compressed more than once.

Compression temperatures ranged from 3 to 5°C, (with two exceptions noted in the text) for the deep animals, and from 6 to 13°C for the shallow water animals. With the maximum biomass utilized, computed oxygen depletion during 60 min resulted in final O_2 levels no lower than 9 mg/l, comparable to water below 250 cm in Lake Baikal in July (Votyntzev, 1961).

Taxonomic identification of all animals tested was carried out along the lines described by Sidelyova (Sidelyova, 1982).

Student's *t*-test was used to assess levels of significance for differences between normally distributed variables. Where the data did not justify the assumption that the results were normally distributed, non-parametric methods were used to estimate likelihood of chance occurrence of differences (Mann–Whitney), or rank correlations (Spearman) (cf. Siegel, 1956). Cut-off in each case was $P \geq 0.05$.

RESULTS

Two of the three families of the suborder Cottoidei of Lake Baikal—cottids (species 1–4, Table 1) and abyssocottids (species 5–10)—were represented in this study. All of the abyssocottids captured (except for a stray specimen of *Asprocottus herzensteini* at 150 m) were found at depths beyond 300 m, 90% of them at depths in excess of 600 m. By contrast, among cottids only *Batrachocottus nikolskii* was restricted to waters of 600 m or more, while the remainder came from shallow or littoral waters.

The shallow water cottids were shorter and lighter than the abyssocottids ($t = 8.9$, $P < 0.001$) (Table 1, columns 5 and 6). The *Batrachocottus* specimens caught were relatively large; their length/weight re-

Table 1. Characterization of Baikalian cottoid species captured

Family	No.	Species name	*N*	Capture depth (*D*) (m)	Total length (*L*) (cm)	Body weight (*W*) (g)
Cottidae						
	1	*Cottus kessleri*	6	45 (15–75)	7.2 ± 1.6	5.5 ± 1.6*
	2	*Cottocomephorus grewingki*	1	75		
	3	*Paracottus kneri*	7	23 (15–45)		
	4	*Batrachocottus nikolskii*	3	870 (600–1000)	13.5 ± 1.1	27.5 ± 1.4†
Abyssocottidae						
	5	*Asprocottus herzensteini*	1	150	9.8 ± 1.2	10.9 ± 2.0*
	6	*Asprocottus abyssalis*	4	608 (485–980)		
	7	*Limnocottus pallidus*	2	1000 (1000)		
	8	*Abyssocottus korotneffi*	8	1043 (485–1400)		
	9	*Cottinella boulengeri*	9	648 (485–1000)		
	10	*Neocottus wereschchageni*	2	950 (900–1000)		

*Regressions of W on L: (1) For spp. 1–3: $W = 0.095 \cdot L^{2.07}$; $r = 0.66$. (2) For spp. 5–10: $W = 0.022 \cdot L^{2.70}$; $r = 0.79$.
†W for sp. 4 predicted from L using: (1) 19.4 g; (2) 25.2 g.

lations conformed more closely to those of abyssocottids than of shallow water cottids (Table 1, footnote).

Subjected to progressively increasing hydrostatic pressures, these fish underwent a regular sequence of changes in locomotor behavior, most completely expressed in the case of abyssocottids. Four succeeding stages were recognizable: (1) The "recovery sequence": resumption of normal vertical orientation of the dorso-ventral axis, resumption of active swimming, substantial periods of time spent off the bottom, and active evasion of a light beam projected into the chamber by fish which at 1 ata were sluggish and frequently poorly oriented [observed in 62% of the abyssocottids (average onset pressure 89 ± 31 ata), but absent in all of the cottids]. (2) Increased activity: increased proportion of time spent swimming actively, and increased rate of swimming, seen in cottids and abyssocottids. (3) Changes in locomotor behavior leading up to, and including, high pressure convulsions. This begins with isolated twitches of tail or fins and progresses, usually fairly sharply, to brief (3–5 sec) but repeated generalized seizures, involving disruption of locomotor behavior, rhythmic whole body contractions, writhing motions, repetitive tail and fin twitches, and violent and disoriented locomotor behavior so that the fish are propelled across the aquarium. In 80% of all animals, transition from isolated twitches to convulsions was sharp and seizure onset pressures (P_c) could be defined ±3–4 ata. In the remainder, the uncertainty was larger but did not exceed ±6 ata. (4) At still higher pressures, motor activity subsided gradually, and eventually the animals became rigid or paralyzed, resting immobile on the bottom and listing to one side or the other; 93% of the shallow water cottids, but only 50% of the abyssocottids, reached this stage. Mean onset pressures in those animals that did show it were: 183.4 ± 51.8 ata for shallow water cottids, and 246.3 ± 32.4 ata for abyssocottids, ($P < 0.05$). The pressure interval separating the onset of this paralysis stage from the seizure onset pressure averaged 100.2 ± 53.6 ata for the cottids, and 51.1 ± 23.9 ata for the abyssocottids ($P < 0.025$). Thus, whatever differentiates the shallow water cottids from the abyssocottids results in greater changes in convulsion threshold pressures than in onset pressures for Stage 4.

Paralysis is presently followed by suspension of respiration, exaggerated secretion of mucus, and death.

The convulsion frequencies for the shallow water cottids constitute a homogeneous unimodal frequency distribution with a mean $P_c = 87.8 \pm 19.6$ ata. The abyssocottids yield a P_c distribution which fails to reveal any clearly defined modal threshold pressure, but suggests that threshold pressures for this group on the whole are higher than for the shallow water cottids. The data for *Batrachocottus nikolskii* occupy an intermediate position between these two distributions (Fig. 1a).

The probabilities of chance occurrence of the several pairs of groups of P_c values, estimated by rank analysis, are: for abyssocottids and shallow water cottids, $P < 0.002$; for *Batrachocottus* and shallow water cottids, $P = 0.02$; for abyssocottids and *Batrachocottus*, $P < 0.05$ (Table 2, footnote, A vs B, A vs C, B vs C). Thus, the data established that the P_c distributions for abyssocottids and for shallow water cottids are different, and make it unlikely that the *Batrachocottus* data could have been drawn from either of these populations.

Could these different P_c distributions for abyssocottids and for cottids result from differences in test temperature? All but two abyssocottids were tested at chamber temperatures between 3 and 4.5°C, close to

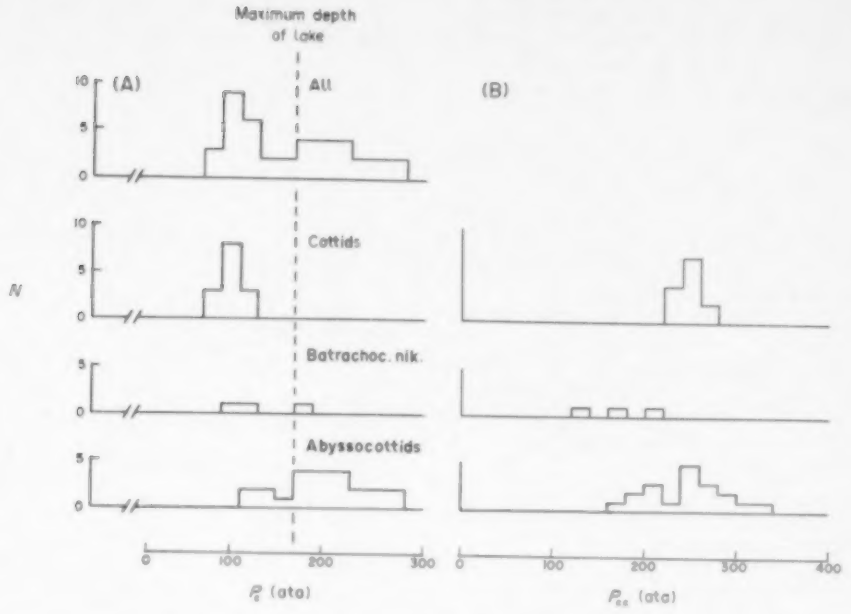

Fig. 1. Frequency distribution diagrams, (A) for convulsion threshold pressure (P_c), and (B) for capture depth corrected convulsion threshold pressure (P_{cc}) (hypothetical common capture depth = 1250 m). Top to bottom: all species; shallow water cottids; *Batrachocottus nikolskii*; abyssocottids.

their habitat temperatures. Two experiments at test temperatures of 7–11°C yielded convulsion threshold pressures of 108 and 125 ata, at the lower end of the distribution for the remaining abyssocottids. Since there is reason to assume that abyssal Baikalian fishes will be stenothermal at or near 3°C, and since previous experiments with another species of fish showed that convulsion threshold pressures for fish are maximal at the acclimation temperature and may decrease when temperatures differ by more than 5°C from the acclimation temperature in either direction (Beaver and Brauer, 1981), these two values were omitted from consideration. The remaining convulsion threshold pressures for the abyssocottids, then, can be taken as representing optimal temperature conditions for these individuals.

Test temperatures for the shallow water cottids ranged from 6 to 13°C, while probable acclimation temperatures at the time of capture of these animals were in the neighborhood of 5–7°C. Despite this range of test temperatures, convulsion threshold pressures for the shallow water cottids varied but little among themselves (Fig. 1A). Test temperature and convulsion threshold pressure in this series showed a Spearman correlation coefficient of −0.23, and $P > 0.4$ that the data represent a chance distribution, indicating that the variation in test temperatures did not measurably affect the observed P_c. The convulsion threshold pressures here reported for the cottids thus are not likely to differ significantly from the optimal convulsion threshold pressures for these animals with respect to temperature. The observed P_c difference between abyssocottids and cottids thus is not attributable to differences in test temperature.

Table 2. Comparison of mean convulsion threshold pressures (P_c) and mean corrected convulsion threshold pressures (P_{cc} for capture depth 1250 m)

	Species	N	$P_c \pm$ stdm	(σ^2)	$P_{cc} \pm$ stdm	(σ^2)
A	Shallow water cottids (A1 + A2)	13	83.4 ± 10.1	(102.1)	243.4 ± 11.3	(127.7)
A	1. *Cottus kessleri*	6	86.9 ± 10.8	(116.6)	246.9 ± 10.9	(118.8)
	2. *Paracottus kneri*	7	79.4 ± 7.3	(52.6)	236.9 ± 7.8	(60.8)
B	Abyssocottids (B1 + B2 + B3)	19	192.7 ± 57.0	(3249)	249.2 ± 39.6	(1568.2)
B	1. *Asprocottus abyssalis*	4	174.7 ± 25.4	(645.2)	257.2 ± 49.3	(2403)
	2. *Cottinella boulengeri*	8	204.5 ± 69.5	(4830)	242.1 ± 29.9	(894)
	3. *Abyssocottus korotneffi*	7	189.4 ± 50.7	(2570)	252.4 ± 41.4	(1714)
C	Batrachocottus nikolskii	3	118.9 ± 36.0	—	167.8 ± 27.6	—

Statistical analysis

	For Δ P_c			For Δ P_{cc}						
Comparing:	A,B	A,C	B,C	A,B	A1, A2	B1, B2	B1, B3	B2, B3	A, C	B,C
t or (U)	(12)	(3)	(7)	0.54	2.2	0.68	0.19	0.55	(3)	(0)
d.f. or N1;N2	19;13	13;3	19;3	32	13	12	11	15	13;3	19;3
P	< 0.002	< 0.02	< 0.05	> 0.4	< 0.05	> 0.4	> 0.4	> 0.4	< 0.001	< 0.01

Linear regression analysis for convulsion threshold pressure on body weight for the cottids showed a correlation coefficient of 0.21, corresponding to $P > 0.2$ for the hypothesis that body weight and convulsion threshold pressure are correlated; the corresponding values for the abyssocottids were $r = 0.35$, $P > 0.05$. Body weight differences within either of the cottoid families studied thus show no significant correlation with convulsion threshold pressures, and differences in group mean body weight fail to account for the observed family differences in convulsion threshold pressures.

Since differences in test temperature and in body weight are the only identified differences associated with our test procedure, we infer that the differences in the distributions of convulsion threshold pressures between shallow water cottids and abyssocottids reflect real physiologic differences between the two groups.

Unlike the shallow water cottoids, capture depths for the abyssocottids varied over several hundreds of meters (Table 1, column 4). Could differences in capture depths within the abyssocottid group have accounted for the complex P_c distribution characteristic of that group (Fig. 1a)? For 25 abyssocottids, the regression of P_c on capture depth is characterized by a regression coefficient of 0.12 ± 0.002; the regression equation was $P_c = 0.12\,D + 110.62$ (Fig. 2a) (P_c = convulsion threshold pressure in ata, and D = the capture depth in m), with a correlation coefficient of 0.49. Given a group size of 25, the probability that a correlation coefficient of this magnitude could have resulted from chance distribution of the sets of capture depths and P_c values is much less than 0.05. Capture depth thus is very probably a significant factor determing convulsion threshold pressure of abyssocottids: The average of P_c increases by 1.2 ata for every atmosphere of increase in hydrostatic pressure at capture depth.

To what extent could capture depth account for the observed differences between mean P_c of shallow water cottids and abyssocottids? The regression of P_c on D based on the combined data for both abyssocottids and shallow water cottids is described by the equation $P_c = 0.13D + 87.4$ with a correlation coefficient of 0.81. The regression coefficient is virtually the same as that which resulted from evaluation of the abyssocottid data alone. Thus, P_c is correlated with capture depth and its probable regression coefficient could account for the differences between mean P_c for shallow water cottids and abyssocottids.

To test the extent to which the marked differences in the width and character of the distributions of P_c values between abyssocottids and shallow water cottids might be a result of differences in spread of capture depths between the two families, convulsion threshold pressures P_c for all experiments were recalculated to yield estimates of the predicted convulsion threshold pressure, P_{∞}, for each individual on the assumption that all had been caught at an arbitrary common hypothetical depth (1250 m was chosen) using the relation $P_{\infty} = P_c - 0.13 \cdot (D - 1250)$ derived from the regression equation for P_c on D). The recalculation (Fig. 2b) effectively eliminates the net dependence of mean convulsion threshold pressure upon capture depth: the regression coefficient for

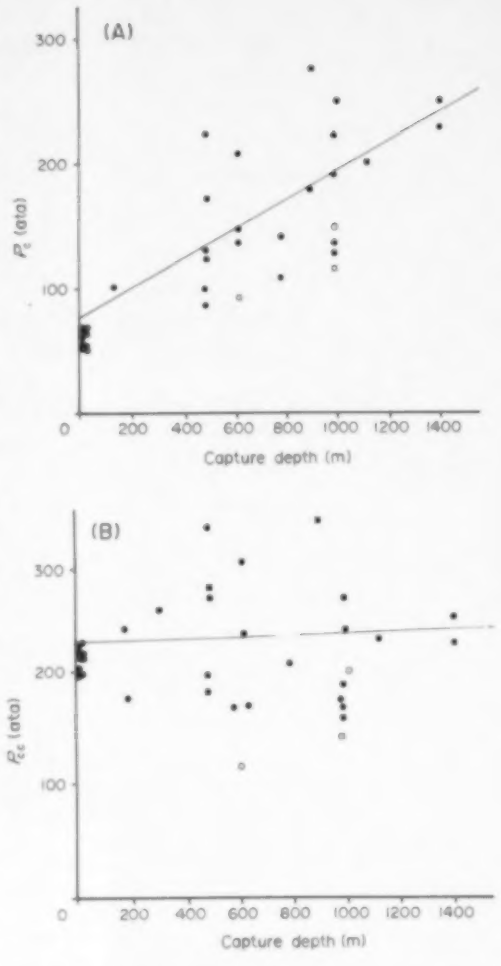

Fig. 2. Correlation diagrams for P_c (A) and P_{∞} (B) with capture depth for all cottoids. Open circles: *Batrachocottus nikolskii*. Shallow water cottids are represented by the cluster of values below 10 ata, all remaining points represent abyssocottids.

P_{∞} on D is 0.009, and mean P_{∞} is 243.1 ± 11.3 ata for shallow water cottids and 249.2 ± 39.6 ata for abyssocottids, ($t = 0.54$, $P > 0.4$ for chance occurrence).

In the following, attention will be focussed upon species represented by four or more individuals in our series (cf. Table 1), i.e. to species 1, 3, 6, 8 and 9, plus *Batrachocottus;* this does not bias the results: mean values for both P_{∞} and P_c computed for the complete and for the restricted sets of cottid and of abyssocottid data, are indistinguishable from one another ($P > 0.2$ for each pair of values).

Transforming P_c to P_{∞} merely raises the mean corrected convulsion threshold pressure of the shallow water cottids by an amount corresponding to the difference between real and hypothetical capture depths without decreasing population variance (Table 2). On the other hand, the distribution curve for P_{∞} for the abyssocottids, in contrast to the corresponding P_c distribution, approaches a normal frequency distribution (Fig. 1B) with a population

variance about half as large as that for the P_c distribution (Table 2, row B). Despite this decrease in population variance, the variance of P_{cc} remains far wider (12.2:1) for the abyssocottids than for the shallow water cottids (Table 2, rows A and B).

Comparison of mean P_{cc} values and standard deviations calculated separately for each of the species show that the small species differences in P_{cc} among the shallow water cottids (Table 2, footnote, A1 vs A2), have little effect on compound population variance of this combined family ($\sigma^2 = 127.1$ for combined group, and 118.8 and 60.8 for the two constituent species). For the three abyssocottid species, mean P_{cc} values are altogether indistinguishable (Table 2, footnote, B1 vs B2, B2 vs B3, B1 vs B3), and the population variances for the individual species bracket the variance for the combined group (Table 2, row B vs B1, B2 and B3, last column), but for all abyssocottid species are far larger than for any shallow water cottid grouping. Thus, the large population variance of P_{cc} values among abyssocottids cannot be accounted for by within-family species differences, and the differences between abyssocottids and cottids in this respect must represent a real difference between our samples of the two cottid families.

Since all *Batrachocottus* specimens were captured in deep water, it is of interest to note the effect of recalculating their convulsion threshold pressures to the common hypothetical capture depth: rank analysis yields $P < 0.01$ that P_{cc} values for *Batrachocottus* were drawn from the same population as the abyssocottids, and $P < 0.001$ that they were drawn from the shallow water cottid population. Thus, the P_{cc} data from which the capture factor is removed indicate, even more strongly than the original P_c data (Table 2, footnote), that *Batrachocottus nikolskii* is a species the pressure tolerance of which sets it apart from both, the family of Cottidae to which it belongs taxonomically, and the family of Abyssocottidae, the habitat of which it shares.

DISCUSSION

Lake Baikal has been in existence as an isolated body of water in eastern Siberia since early tertiary times, and as a deep body of water at least since the early Pleistocene (Kozhov, 1972). The suborder of cottoid fishes is represented there by three highly endemic families (Taliev, 1955; Sidelyova, 1982), two of which (Cottidae and Abyssocottidae) are of interest here because the majority of the members of each reside in rather well defined depth zones within the lake—cottids in shallow, and abyssocottids in deep water. Habitat depth for specific adults can be inferred with reasonable confidence from trawl data: these are relatively inactive benthic or benthopelagic fishes (Sidelyova, 1982), and bottom contours of the deeper parts of Lake Baikal are relatively flat (Lut, 1978) so that capture depths and habitat depths are unlikely to differ greatly from one another. Each of these two families includes a few species which, presumably secondarily, have established themselves at depths not normally inhabited by adults of other species of their family—deep dwelling *Batrachocottus* species among cottids, and shallow water dwelling *Procottus* species among abyssocottids (Sidelyova, 1982), permitting comparisons of shallow and deep water adaptation not only between species belonging to different families of a common suborder, but also within each family. It was hoped that this unique situation might permit one to assess for the first time the relative roles of acclimation as compared to (genetically determined) adaptation of deep water animals to a high pressure environment: in Lake Baikal, pressures range from 1–2 ata in the littoral zone, to 140–160 ata at the bottom of the deep basins (Lut, 1978). Since most surface dwelling vertebrates (including fish (Beaver and Brauer, 1981)) undergo substantial disturbances of locomotor behavior when compressed to pressures below 100 ata (Brauer *et al.*, 1974), deep dwelling fish species in Lake Baikal most probably will have to show some degree of acclimation (or adaptation) to high pressures. Since cottoids as a group are devoid of a swim-bladder (Berra, 1981), direct experimental test of pressure tolerance of individual fishes, by observing behavior during compression, was feasible and confirmed the hypothesis: shallow water cottids undergo high pressure convulsions at an average pressure of 87.8 ata, while abyssocottids convulse at pressures from 120 to 270 ata, 2/3 of them showing seizures only at pressures exceeding the 160 ata, prevailing in the deepest parts of Lake Baikal (Fig. 1A).

Additional evidence for acclimation or adaption to high pressures is to be found in the "recovery stage", shown by most abyssocottids during the early stages of compression (really recompression). A similar stage was observed by Childress *et al.* (1980) in meso- and abyssopelagic Pacific fishes, as well as by several workers in deep water crustaceans (Brauer *et al.*, 1983; Mickel and Childress, 1982; Macdonald and Gilcrist, 1982; Yayanos, 1978) and implies that for these deep water animals decompression to 1 ata represents a stress from which they recover promptly when the pressure is raised to levels near those characteristic of their habitat depths.

While high hydrostatic pressures affect a wide variety of systems (Macdonald, 1975), among vertebrates central nervous system-related changes pre-dominate in the pressure range of interest here (Rostain, 1980; Bennett and Dossett, 1973; Balouet *et al.*, 1973; Brauer *et al.*, 1971), and onset of high pressure convulsions is thus likely to prove a meaningful index of the degree of pressure tolerance in these fishes.

The distribution of P_c, the convulsion onset pressure, reveals a clear-cut separation of two major groups along taxonomic lines as described by Sidelyova (1982): shallow water cottids show a narrow range of low convulsion threshold pressures clearly distinct from the wide distribution of high threshold pressures for abyssocottids (Fig. 1A). *Batrachocottus nikolskii* (one of the few deep water cottid species) occupies an intermediate position, probably distinct from that of either of the two major family groups (Table 2, footnote, A vs B, and A or B vs C).

Since prolonged exposure to high pressure environments can induce a measurable increase in pressure tolerance of both crustaceans (Roer *et al.*, 1980) and mammals (Hinson and Brauer, unpublished), and since the slope of the regression of observed P_c on

capture depth for these cottids very clearly corresponds to the rate of increase of hydrostatic pressure with depth, one might conclude that the present data merely establish that, not unexpectedly, these fish are acclimated to the hydrostatic pressures of their usual habitat.

Two factors seem to us to militate against such an interpretation. In the first place, the distributions of P_c values for the shallow water cottids and for the abyssocottids reveal widely different degrees of variability not accounted for by within-family species differences or differences in the range of capture depths (cf. 12.2:1 variance ratio for P_{cc}, Table 2, column 6). Explanations proposing to attribute this difference to decompression trauma or to a substantial degree of eurybathiality of abyssocottids seem to us improbable; the former because of the prompt and dramatic recovery of these fish upon recompression, the latter because of the essentially benthic lurking mode of life of all adult abyssocottids (cf. Taliev, 1955), together with the known bathymetric characteristics of the deep lake bed (Lut, 1978). A more likely interpretation seems to us that the high population variances in P_c and P_{cc} among abyssocottids reflect lack of selection pressure for this characteristic since these pressure tolerances exceed the greatest pressures encountered in the lake, and thus functionally distinguish this family from that of the cottids.

A second argument bearing the nature of the observed differences in pressure tolerance among Baikalian cottoids is observed from the observations that in *Batrachocottus nikolskii*, unlike the remaining species, mean convulsion threshold pressures (P_c or P_{cc}) exceed habitat pressures by less than 45 ata (Table 2). Analysis of both raw and capture depth compensated convulsion threshold pressures showed it to be exceedingly unlikely that even our limited sample of *Batrachocottus nikolskii* could have been drawn from either the shallow water cottid or the abyssocottid populations (Table 2, footnote, A vs C, and B vs C for P_c and P_{cc}). This species thus occupies a special intermediate place with respect to our criteria, corresponding neatly to the special role assigned to it on taxonomic and ecologic grounds (Sidelyova, 1982), a place which cannot readily be explained without assuming a substantial degree of genetic derminacy to account for the limited pressure tolerance brought by this species from its shallow water ancestors to its present deep water habitat. Such a conclusion would be in keeping with the demonstration that in mice genetic factors can indeed determine pressure tolerance to a marked degree (McCall and Frierson, 1981).

Taken together, the family differences in population variance of P_{cc} and the special position of *Batrachocottus nikolskii*, seem to us to make a good case for the hypothesis that differences in pressure tolerance in Baikalian cottoid species are in considerable measure adaptive rather than acclimational in character. If borne out by further work (especially involving *Procottus*, the second "cross-over" genus), this would represent the first clear-cut demonstration that hydrostatic pressure as such is a significant selection factor capable of affecting the evolution of deep water vertebrate species.

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STUDIES ON THE ABSORPTION OF LIPID FROM THE GUT OF DESERT LOCUST, *SCHISTOCERCA GREGARIA*

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Abstract—1. Absorption of ingested radioactive lipids from the gut of *Schistocerca gregaria* was studied *in vitro*.

2. Analysis of the absorbed lipids showed that the lipids are absorbed mainly as diacylglycerol.

3. Electrophoretic analysis of the incubated haemolymph medium revealed that the absorbed radioactive lipids are associated with a haemolymph lipoprotein.

4. Incubation medium lacking in haemolymph altogether, also revealed a labelled lipoprotein which has the same electrophoretic mobility as the haemolymph lipoprotein.

5. It is suggested that the lipids are absorbed from gut as lipoproteins.

INTRODUCTION

Until recently very little was known about the manner in which ingested lipids were absorbed from the gut of insects. Recent studies by Weintraub and Tietz (1973, 1978) indicated that in the locust, *Locusta migratoria*, ingested radioactive triacylglycerol was first hydrolysed in the gut lumen mostly into fatty acids and glycerol, which in turn were reesterified in the midgut wall and finally entered the haemolymph mainly as diacylglycerol. In the cabbage butterfly, *Pieris brassicae*, Turunen (1975) demonstrated *in vivo* that diacylglycerol was the major lipid metabolite that entered the haemolymph from the gut. On the other hand, Reisser-Bollade (1976) claimed that in the cockroach, the ingested lipids were absorbed mainly as triacylglycerol. More recently, Chino and Downer (1979) disputed this finding and presented evidence that diacylglycerol was the principal class of lipid absorbed in this insect also. These studies thus indicated that lipids are absorbed from the gut of insects primarily as diacylglycerol. In all of these studies, except the one by Weintraub and Tietz (1978), intact insects were used and therefore it is difficult to ascertain fully whether the radioactive diacylglycerol present in the haemolymph was indeed released from the gut, or if it might have originated from fatbody, since fatbody is an important source of haemolymph diacylglycerol in insects (Gilbert, 1967; Gilbert and Chino, 1974). This problem can be solved only by employing isolated alimentary canal *in vitro*. Weintraub and Tietz (1978) studied lipid absorption by the isolated gut of *Locusta* and the results of their *in vitro* studies support the *in vivo* studies that diacylglycerol is the principal lipid absorbed from the gut.

This work was undertaken to investigate lipid absorption in *Schistocerca gregaria* with exclusively *in vitro* systems of isolated alimentary canal from locusts previously fed with a variety of radioactive lipids. The results presented here provide more definite evidence that diacylglycerol is the principal lipid absorbed from the midgut of this insect and that the lipid is released from the gut as lipoproteins.

MATERIALS AND METHODS

Animals

Desert locusts (*Schistocerca gregaria*) were raised on fresh endive or fresh grass and dry wheat bran at 30°C under constant light. Both male and female locusts as well as last-instar nymphs were used at random. Adults were used approximately 5–10 days after the last moult.

Chemicals

[Carboxy-^{14}C]trioleylglycerol (specific activity 30 μCi/μmol), [U-^{14}C]glycerol (specific activity 25 μCi/μmol), [1-^{14}C]palmitic acid (specific activity 50 μCi/μmol) and [9,10(n)-^{3}H]oleic acid (specific activity 2 Ci/μmol) were purchased from Amersham Corporation, Oakville, Ontario. Standard lipid samples were obtained from Applied Sciences Laboratories, State College, Pennsylvania. All organic solvents used were glass distilled and were of analytical grade.

Feeding of labelled compounds

Locusts were starved overnight prior to feeding. They were force-fed individually with 50 μl of the radioactive lipids as emulsions using a microsyringe as follows: 5 μCi of [carboxy-^{14}C]trioleylglycerol mixed with 2 mg cold trioleylglycerol, 5 μCi of [^{14}C]palmitic acid plus 1 mg cold 2-mono-oleylglycerol, 5 μCi of [9,10(n)-^{3}H]oleic acid mixed with 1 mg cold oleic acid plus 1 mg 2-mono-oleylglycerol or 5 μCi of [U-^{14}C]glycerol mixed with 2 mg cold oleic acid or palmitic acid. These mixtures were emulsified prior to feeding by suspending them in 0.25 ml of locust saline containing 5% bovine serum albumin, 0.25% glycerol and 0.125% Tween 80, and sonicating for 1 min at room temperature. The emulsion was stable for 2 weeks. The locust was held with one hand on the wings and large legs with ventral side up in a horizontal position and measured drops of the emulsion were allowed to touch the oral cavity of the locust. The emulsion was taken up readily by locusts, which then were transferred to glass containers containing fresh endive and kept for 30 min before the gut was removed for incubation.

Preparation of alimentary canal for incubation

About 30 min after ingestion of radioactive emulsion, the alimentary canal of the locust was exposed and freed from trachea and fatbody. The entire gut was removed carefully with the head and the anal tergum still attached to the gut. The isolated gut preparation was placed in a paraffin-lined petri dish, rinsed thoroughly with locust saline and examined under a dissecting microscope. Care was taken to remove any body fat sticking to the gut, and the entire gut surface was observed for any damage. If damage was noticed, the gut was discarded. The isolated gut was transferred to a 10 ml-beaker containing 2.0 ml of a suspension medium containing either 0.3 ml of locust haemolymph plus 1.7 ml of locust Ringer or 2 ml of locust Ringer alone. The alimentary canal was immersed in the incubation medium by suspending it from the rim of the beaker with two insect pins bent into an "S"-shaped hook, one inserted through the oral end of the head and the other through the anal tergum. In this way only part of the crop, but the entire gastric caeca and midgut will be completely immersed in the incubation medium. Such a preparation when examined under the microscope exhibited regular rhythmic movements. The gut was incubated in a water-bath at 32°C with gentle shaking for a period of 1–2 hr. During a few initial experiments, the incubation medium was aerated with a gentle stream of O_2 and CO_2. This was found later to be unnecessary as no apparent difference in the radioactivity of released lipids was noticed with or without oxygen. During the course of incubation, the condition of the gut was checked periodically under the microscope by observing the motility of the midgut and gastric caeca. Even after 2 hr of incubation, the gut remained viable and exhibited definite movement. At the end of the incubation, the midgut along with the gastric caeca were severed from the rest of the gut.

Extraction and separation of lipids

Lipids from the incubation medium were extracted by homogenization in chloroform–methanol (2:1 v/v) in a Sorvall Omnimixer with subsequent filtering and washing (Folch *et al.*, 1957). The midgut wall was homogenized after washing out the midgut contents with fine sand in 0.5 ml of locust Ringer and the homogenate was subsequently extracted for lipids as above. Lipids from the midgut contents were likewise extracted with chloroform–methanol mixture. The neutral lipids were chromatographed on Silica Gel G TLC plates in either hexane–diethyl ether–acetic acid (80:20:1.5 v/v) or diethyl ether–benzene–ethanol–acetic acid (40:50:2:0.2 v/v). Lipid fractions were visualized on the TLC plates by exposing the plate to iodine vapours and each lipid class was identified by comparison with known marker lipids.

Radioassay

After visualization of the lipid spots on the TLC plate with iodine, each spot was marked, scraped off the plate and transferred directly to scintillation vials by means of a manual vacuum plate scraper (Mandel Scientific Co., Rockwood, Ontario). Ten millilitres of scintillation fluid (4 g PPO and 0.1 g POPOP in 1 l toluene) were added to each vial and radioactivity was counted with a Beckman Liquid Scintillation spectrometer (Model LS-133), equipped with an automatic external standard.

Disc electrophoresis

Aliquots from the incubation medium containing haemolymph were used as such for electrophoresis. When the incubation medium contained only locust Ringer, it was concentrated to a known volume (100–200 μl) prior to electrophoresis by dialysing against Aquacide-II (Calbiochemicals, Los Angeles, California). Disc electrophoresis was carried out as described previously (Thomas, 1979). Some of the incubation media were prestained with 1%

Table 1. Adsorption of fed [^{14}C]triolelylglycerol from the alimentary canal of *Schistocerca gregaria in vitro*

Lipid fraction	Released [^{14}C]-lipids into haemolymph medium		[^{14}C]-lipids incorporated into midgut wall		[^{14}C]-lipids in the midgut contents	
	Radioactivity (counts/min × 10^{-2})	% Label released	Radioactivity (counts/min × 10^{-2})	% Label incorporated	Radioactivity (counts/min × 10^{-2})	% Label incorporated
Triacylglycerol	7.701 ± 4.410* (9)	5.51 ± 3.00*	110.14 ± 25.47* (5)	35.84 ± 8.06*	25.69 ± 16.36* (4)	8.03 ± 3.08*
Diacylglycerol	99.432 ± 20.520 (9)	75.54 ± 8.84	31.09 ± 15.55 (5)	9.16 ± 2.80	17.96 ± 3.13 (4)	4.94 ± 0.51
Monoacylglycerol	4.653 ± 2.452 (9)	3.72 ± 2.25	6.08 ± 3.95 (5)	2.05 ± 0.97	8.36 ± 3.37 (4)	2.21 ± .56
Non-esterified fatty acids	13.362 ± 10.408 (9)	8.10 ± 3.84	35.77 ± 15.13 (5)	11.22 ± 5.91	296.90 ± 99.42 (4)	79.06 ± 9.39
Phospholipids	11.215 ± 3.365 (9)	7.13 ± 1.09	162.56 ± 81.47 (5)	41.72 ± 7.33	20.05 ± 3.04 (4)	5.76 ± 2.09

The locust was fed with 1 μCi of [1-^{14}C]triolelylglycerol as emulsion. The gut was removed after 30 min and incubated in a medium containing 2.0 ml of locust saline + haemolymph for 60 min. After incubation lipids were extracted from the medium, midgut wall and midgut contents, fractionated by TLC and radioassayed.
*Mean ± SEM (number of determinations).

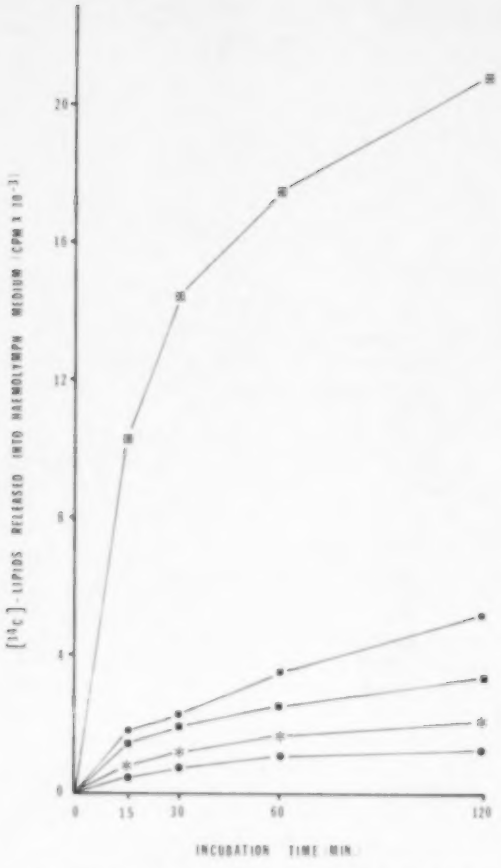

Fig. 1. Time course of the absorption of fed [1-^{14}C]trioleylglycerol from the gut of *Schistocerca gregaria in vitro*. Locust was fed 1.5 μCi of [1-^{14}C]trioleylglycerol as emulsion. The gut was removed after 30 min and incubated in a medium containing 1.7 ml locust saline and 0.3 ml locust haemolymph for various periods of time. Aliquots of 0.5 ml of the medium were withdrawn at specific times and replaced by 0.5 ml fresh medium. ●——●, [^{14}C]Diacylglycerol; ★——★, [^{14}C]nonesterified fatty acids; ■——■, [^{14}C]phospholipids; ✱——✱, [^{14}C]triacylglycerol; and ●——●, [^{14}C]monoacylglycerol.

(w/v) Sudan IV in diethylene glycol prior to disc electrophoresis (Thomas and Gilbert, 1967). Tris–glycine buffer, pH 8.6, 4% polyacrylamide and a current of 2.0 mA/tube were used. After electrophoresis, the gels were stained with 0.5% Aniline blueback in 7% acetic acid for proteins. Radio-assay of the prestained lipoprotein bands from the gels after electrophoresis was performed according to Basch (1968).

RESULTS

Absorption of ingested [carboxy-^{14}C]trioleylglycerol from the isolated gut

The distribution of radioactivity in lipids released from gut incubated in the haemolymph containing medium is shown in Table 1. The diacylglycerol accounted for about 76% of the radioactivity of the lipids released into the incubation medium, while the non-esterified fatty acids comprised less than 5% and the phospholipids about 8% of the total radioactivity of the released lipids. The time course of the release of [^{14}C]diacylglycerol and other lipids in the incubation medium is shown in Fig. 1. The release of diacylglycerol continued to increase with time throughout the 2-hr incubation period. The release of other lipids remained relatively unchanged except for the non-esterified fatty acids and phospholipids that exhibited a slight increase with time. The distribution of radioactivity of the mid-gut wall lipids and the lipids of the midgut contents is shown in Table 1. The ingested triacylglycerol is hydrolysed in the gut lumen into mostly free fatty acids (Table 1). About 80% of the radioactivity of the lipids of the gut contents was associated with the free fatty acids. This indicates that lipolysis is almost complete in the gut lumen. In the gut wall, however, the distribution of radioactivity in lipids changed almost entirely from that of the gut contents (Table 1). This indicates that some metabolic changes took place after the lipids were absorbed into the gut wall from the lumen. About 41% of the radioactivity of the gut wall lipids was associated with the phospholipid fraction, while the triacylglycerol accounted for about 36% and the diacylglycerol only about 10% of the total radioactivity.

Absorption of ingested [^{3}H]oleic acid from the isolated gut

In order to test whether ingested non-esterified fatty acids would follow the same esterification steps and release as ingested triacylglycerol, locusts were fed with [^{3}H]oleic acid. It can be seen from Table 2 that the ingested oleic acid was readily absorbed by the gut wall and metabolized mainly into triacylglycerol, phospholipids and diacylglycerol. Unlike locusts fed on the [^{14}C]trioleylglycerol in which gut wall lipids showed more radioactivity incorporated into phospholipids than triacylglycerol, in the locusts

Table 2. Absorption of fed [^{3}H]oleic acid from the alimentary canal of *Schistocerca gregaria in vitro*

	Released [^{3}H]-lipids into haemolymph medium		[^{3}H]-lipids incorporated into midgut wall	
Lipid fraction	Radioactivity (counts/min × 10^{-2})	% Label released	Radioactivity (counts/min × 10^{-2})	% Label incorporated
Triacylglycerol	7.513 ± 2.45* (6)	3.23 ± 1.06	283.306 ± 65.24 (5)*	50.09 ± 7.03
Diacylglycerol	187.301 ± 37.46 (6)	81.03 ± 7.82	72.480 ± 34.02 (5)	12.37 ± 4.48
Monoacylglycerol	5.601 ± 1.75 (6)	3.08 ± 1.47	5.358 ± 1.81 (5)	1.12 ± 0.33
Non-esterified fatty acids	16.220 ± 11.27 (6)	7.07 ± 4.31	42.770 ± 20.61 (5)	7.28 ± 4.06
Phospholipids	11.306 ± 5.61 (6)	5.59 ± 3.22	160.71 ± 41.71 (5)	29.14 ± 8.40

The locust was fed with 1 μCi of [^{3}H]oleic acid as emulsion along with 5 mg monoleylglycerol. Other conditions are as in Table 1.
*Mean ± SEM (number of determinations).

Table 3. Absorption of fed [^{14}C]glycerol from the alimentary canal of *Schistocerca gregaria in vitro*

Lipid fraction	Released [^{14}C]-lipids into haemolymph medium		[^{14}C]glycerol incorporated into midgut wall lipids	
	Radioactivity (counts/min × 10^{-2})	% Label released	Radioactivity (counts/min × 10^{-2})	% Label incorporated
Triacylglycerol	7.102 ± 2.401* (4)	7.80 ± 2.29	217.24 ± 80.03 (4)*	33.91 ± 4.75
Diacylglycerol	61.269 ± 27.190 (4)	78.98 ± 1.49	107.03 ± 23.69 (4)	16.78 ± 1.82
Monoacylglycerol	3.367 ± 0.915 (4)	3.27 ± 1.11	10.26 ± 7.18 (4)	1.51 ± 1.11
Phospholipids	10.860 ± 3.517	9.95 ± 2.12	307.59 ± 161.63 (4)	47.80 ± 4.59

The locust was fed with 1 μCi of [^{14}C]glycerol and 5 mg of oleic acid as emulsion. Other conditions are as in Table 1.
*Mean ± SEM (number of determinations).

fed [^{3}H]oleic acid 50% of the radioactivity in gut wall lipids was present as triacylglycerols, while the phospholipids had only about 29% of the label. However, here again as in the [^{14}C]trioleylglycerol fed locust, diacylglycerol accounted for about 81% of the radioactivity in the total lipids released into the haemolymph medium. Thus, esterification and release of ingested lipids followed a similar metabolic course in both cases.

Absorption of [^{14}C]glycerol by the isolated gut

Because both the ingested triacylglycerol and oleic acid were absorbed chiefly as diacylglycerol, it was decided to test whether ingested glycerol would undergo direct esterification into glycerides by the midgut wall and would also be released into the incubation medium mainly as diacylglycerol. [U-^{14}C]glycerol was fed to the locust along with non-labelled oleic acid as an emulsion. Even though the greater part of the ingested glycerol was absorbed as free glycerol, our results show that part of the ingested glycerol did indeed undergo esterification and was metabolically converted into glycerides and phospholipids in the gut wall, while the lipids released into the haemolymph incubation medium were primarily diacylglycerols (Table 3). It can be seen that the distribution of the label among the lipids released into the medium from the gut here did not differ very much from that of either the [^{14}C]trioleylglycerol fed locust gut or the [^{3}H]oleic acid fed locust gut.

Release of lipids from isolated gut into incubation medium devoid of haemolymph

In order to test whether any release of lipid from the isolated gut would occur into a medium devoid of haemolymph, gut from locusts fed either with [^{14}C]trioleylglycerol or [^{3}H]oleic acid was incubated in a medium containing only locust saline. The results of these studies are shown in Table 4. It can be seen that lipid release is not interrupted from the gut even when haemolymph was excluded from the incubation medium. In fact, in both cases the distribution of the label among the lipids released into the incubation medium followed the same pattern as that observed among the lipids released into medium containing haemolymph (Tables 1 and 2).

Electrophoresis of incubation media

Both the haemolymph-containing incubation medium as well as the medium devoid of haemolymph were subjected to disc electrophoresis. A definite lipoprotein band is clearly present in both incubation media (Fig. 2). The radioactivity of these lipoprotein bands is shown in Table 5. The lipoprotein band visible in the haemolymph-free incubation medium migrated the same distance as the lipoprotein band from the haemolymph as well as the lipoprotein band from the haemolymph-containing incubation medium. Gels stained with aniline blue-black for proteins revealed several protein bands in addition to the lipoprotein band in the haemolymph-containing medium, while in the haemolymph-free incubation medium the major protein band visible was the lipoprotein band, and the other protein bands were fewer and fainter.

DISCUSSION

The results of this study agree for the most part with those reported in previous investigations (Weintraub and Tietz, 1973, 1978; Turunen, 1975; Chino and Downer, 1979) that the ingested lipids are absorbed mainly as diacylglycerol. Almost all of the above studies employed *in vivo* procedures utilizing

Table 4. Release of fed [1-^{14}C]trioleylglycerol and [^{3}H]oleic acid into haemolymph-free locust saline medium from the alimentary canal of *Schistocerca gregaria in vitro*

Lipid fraction	Released [^{14}C]-lipids into locust saline medium		Released [^{3}H]-lipids into locust saline medium	
	Radioactivity (counts/min × 10^{-2})	% Label released	Radioactivity (counts/min × 10^{-2})	% Label released
Triacylglycerol	5.10 ± 3.33 (4)*	4.48 ± 3.26	9.502 ± 3.04 (4)	4.37 ± 2.25
Diacylglycerol	94.51 ± 19.09 (4)	78.68 ± 1.50	160.210 ± 29.79 (4)	73.27 ± 8.62
Monoacylglycerol	4.73 ± 2.80 (4)	3.92 ± 2.45	10.122 ± 4.43 (4)	4.48 ± 1.08
Non-esterified fatty acids	6.08 ± 2.31 (4)	4.98 ± 1.52	22.270 ± 13.64 (4)	9.65 ± 5.07
Phospholipids	9.99 ± 5.27 (4)	7.94 ± 2.49	18.072 ± 5.89 (4)	8.23 ± 3.83

Locust was fed with 1 μCi of either [1-^{14}C]trioleylglycerol or [^{3}H]oleic acid as emulsion. The gut was removed after 30 min and incubated in a medium, containing only 2.0 ml of locust saline and no haemolymph. Other conditions are as in Table 1.
*Mean ± SEM (number of determinations).

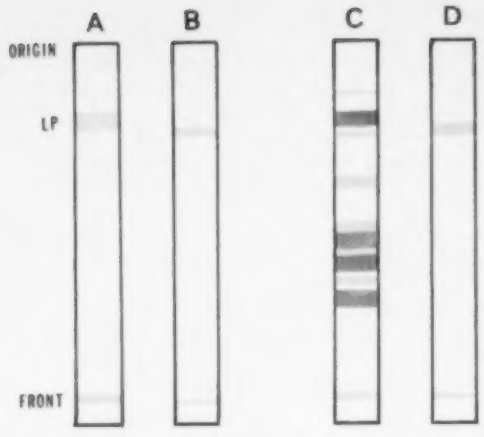

Fig. 2. Schematic diagrams of disc gel electrophoretograms of haemolymph incubation medium and haemolymph-free saline incubation medium, using 4% gel. (A) Haemolymph medium prestained with Sudan IV for lipoproteins; (B) haemolymph-free saline medium prestained with Sudan IV; (C) haemolymph medium stained with Aniline blue-black for proteins; and (D) haemolymph-free saline medium stained with Aniline blue-black. Other conditions are as in Table 5.

intact insects. The only *in vitro* study conducted so far employing isolated alimentary canal has been that reported for *Locusta migratoria* by Weintraub and Tietz (1978). They showed that isolated alimentary canal from locusts which were previously fed with radioactive triacylglycerol when incubated in a medium containing locust haemolymph, released lipid into the haemolymph medium primarily as diacylglycerol.

In the present study, the locusts were fed directly with a known quantity of radioactive lipids as emulsion rather than feeding them with grass on to which radioactive lipids were previously applied. This procedure enabled us to administer a known quantity of the lipid each time when the locust was fed, thus assuring more consistent results. The *in vitro* procedure employed in this study also enables one to monitor the viability and condition of the alimentary canal during the incubation period by microscopical observation for a healthy and living gut will exhibit slow but steady contractile movements.

The data presented in this study would show that no matter in what form the lipid or lipid precursor was ingested by the locust (triacylglycerol, oleic acid or glycerol), the lipid absorbed in all cases had been chiefly as diacylglycerol. Time course of the absorption of ingested [^{14}C]trioleylglycerol by the isolated gut would indicate that among the absorbed lipids, only diacylglycerol release was a continuous and time dependent process, while the release of other lipids exhibited no significant increase with time. As suggested by Turunen (1979), it is possible that diacylglycerol in insects may have functions identical to that of triacylglycerol in mammals in the translocation of lipids across the midgut.

On the basis of their *in vivo* as well as *in vitro* studies, Weintraub and Tietz (1978) suggested that the fatty acids formed in the lumen as a result of the hydrolysis of the ingested trioleylglycerol would enter the midgut cells where they are metabolically incorporated into phospholipids, di- and triacylglycerols, but later released into the haemolymph mainly as diacylglycerol. The results of our study seem to substantiate this contention. In *Schistocerca*, among the gut wall lipids, radioactivity was observed more in the phospholipids and triacylglycerol than in the diacylglycerol. The percentage of label incorporated into phospholipids, di- and triacylglycerol of the midgut wall differed significantly according to the kind of lipid ingested. The data presented in this study as well as those reported by Weintraub and Tietz (1978) suggest the midgut cells as the source of haemolymph diacylglycerol. However, the manner in which it is formed within the midgut cells and the mechanism by which it is released into the haemolymph are largely unknown.

Since the midgut wall lipids contain about 10–20% of the label in the diacylglycerol fraction at any given time, it seems logical to assume that direct esterification of fatty acids into diacylglycerol could be one of the pathways for its formation. We have observed that isolated microsomes of the midgut cells have the ability to esterify monoacylglycerol and fatty acids into diacylglycerol *in vitro* (unpublished observation). Furthermore, the occurrence of monoacylglycerol acyltransferase activity in the midgut of *Periplaneta americana* has been reported by Hoffman and Downer (1979).

Among the gut wall lipids, however, the phospholipids exhibited marked incorporation of the labelled fatty acids. By means of double-labelling experiments, Weintraub and Tietz (1973, 1978) were able to establish a correlation between the phospholipid synthesis in the midgut and diacylglycerol release in

Table 5. Radioactivity of lipoproteins after disc electrophoresis of incubated saline medium and dilute haemolymph medium

Incubation medium	Radioactivity (counts/min × 10^{-2})	
	[^{3}H]palmitic acid	[^{14}C]trioleylglycerol
Haemolymph medium	4.12 ± 1.87* (4)	2.39 ± 1.06 (4)
Haemolymph-free saline medium	11.08 ± 4.18 (5)	7.81 ± 2.78 (4)

Locusts were fed 1 μCi of [1-^{14}C]trioleylglycerol or [1-^{14}C]palmitic acid as emulsion. The gut was removed after 30 min and incubated in a medium consisting of only 2.0 ml of locust saline or in a medium consisting of 2.0 ml locust saline + haemolymph for 60 min. After incubation of labelled gut, the incubation media were subjected to gel electrophoresis. An aliquot of 0.1 ml of the haemolymph was used for electrophoresis, while the saline medium was concentrated to 0.2 ml and 50 μl of this was used for electrophoresis. Both samples were prestained with Sudan IV prior to electrophoresis.

*Mean ± SEM (number of determinations).

Locusta. As a result, they suggested that midgut wall phospholipids may be the source of haemolymph diacylglycerol. On the other hand, the rapid synthesis of phospholipids observed in the midgut mucosa could be a reflection of the rapid membrane synthesis going on in these cells during absorption. Or, as Turunen (1979) suggested, the phospholipid synthesis may be directly concerned with the intracellular lipid movement in the midgut mucosa. The significance of the relatively high radioactivity observed in the midgut wall triacylglycerol is also unclear. It is possible that this triacylglycerol could be another source for diacylglycerol formation in the midgut mucosa or that their synthesis may be associated with some aspects of lipid absorption. The presence of diacylglycerol acyltransferase activity in the midgut of *Periplaneta americana* has been reported recently by Hoffman and Downer (1979). However, the studies to date do not permit any definite conclusions as to the significance of the phospholipid and triacylglycerol synthesis observed in the midgut wall during lipid absorption.

The observation made in this study that a small percentage of the ingested [^{14}C]glycerol was incorporated into the acylglycerols and phospholipids of the midgut wall, but released into the haemolymph primarily as diacylglycerol would seem to indicate that glycerol can serve as a precursor for the synthesis of acylglycerols and phospholipids by the midgut mucosa. However, the metabolic pathways by which acylation of glycerol would take place remain to be determined.

The result of this study also indicate that for the release of diacylglycerol from the incubated gut, the presence of haemolymph in the incubation medium is not necessary, since diacylglycerol release was not interrupted from the gut which was incubated in the medium devoid of haemolymph. In fact, the pattern of lipid release from the incubated gut into haemolymph-free medium was essentially similar to that of the gut incubated in medium containing haemolymph. Electrophoretic analysis of the incubation medium that lacked the haemolymph indeed revealed a lipoprotein band that has more or less the same electrophoretic mobility as the haemolymph lipoprotein band. These observations seem to suggest that the lipids are absorbed as lipoproteins. Furthermore, the lipoprotein band demonstrable in the haemolymph-free incubation medium was the most heavily labelled component of the protein bands. That the lipoprotein band observed in the haemolymph-free medium was indeed the result of lipoproteins released during the incubation from the gut and not appearing as possible contamination by haemolymph adhering to the gut during incubation can be ascertained by the fact that many of the other dense protein bands that are characteristic of the haemolymph in the electrophoretogram were lacking in the haemolymph-free medium. It thus seems evident that the midgut mucosa has the potential for esterifying ingested fatty acids chiefly as diacylglycerol and conjugating them with proteins as lipoproteins prior to their release into the haemolymph. However, experiments such as those reported here are not sufficient in themselves to confirm the finding that the absorbed lipids are released as lipoproteins in the locust. Further experiments employing immunological techniques are currently in progress in order to clarify the above observation. Nevertheless, evidence so far seems to indicate that the fatty acids derived from ingested lipids would undergo esterification within the midgut cells into acylglycerol and phospholipids and that the esterified lipids are released from the gut into haemolymph chiefly as diacylglycerol in the form of lipoproteins.

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A DIABETES INSIPIDUS-LIKE SYNDROME IN THE INTACT YUCATAN MINIATURE BOAR FOLLOWING IMPLANTATION OF DESOXYCORTICOSTERONE-ACETATE (DOCA)

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(*Received* 8 *July* 1983)

Abstract—1. A syndrome of polydipsia and polyuria is described in the intact adult Yucatan miniature boar following implantation of silicone rubber strips impregnated with desoxycorticosterone-acetate (DOCA).

2. Water intake was significantly greater than control 5 days post-DOCA.

3. Urine output was significantly greater than control 7 days post-DOCA, and urine osmolality was significantly decreased 8 days post-DOCA.

4. Serum potassium was significantly less than control 24 hr post-DOCA, and serum sodium was consistently and significantly greater than control by 1 week post-DOCA.

5. As suggested in the dog, the increase in water turnover following DOCA administration in the pig is initiated by an increased thirst followed by an increase in urine output.

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INTRODUCTION

The appearance of polydipsia following the injection of desoxycorticosterone-acetate (DOCA) in oil or water suspensions has been reported in normal dogs on normal or elevated sodium intakes (Ferrebee *et al.*, 1941; Kuhlman *et al.*, 1939; Ragan *et al.*, 1940; Moehlig and Jaffe, 1942; Mulinos *et al.*, 1942), the intact rat (Selye *et al.*, 1943; Selye and Basset, 1940; Rice and Richter, 1943), the chicken (Stamler *et al.*, 1951), and the rabbit, if sodium chloride solutions were substituted for water (Masson *et al.*, 1953; Denton and Nelson, 1970). An attempt to induce polydipsia with DOCA in the cat was unsuccessful (Winter and Ingram, 1943). A transient polyuria was also observed in the intact monkey (Knobil *et al.*, 1953). Studies in the uninephrectomized pediatric pig demonstrated a marked polydipsia and polyuria which developed 4 days following DOCA implantation (Grekin *et al.*, 1980). In a preliminary report Terris and Simmonds (1979) reported the development of polyuria and polydipsia in the intact adult Yucatan miniature boar following the administration of DOCA or aldosterone implants.

Although the precise mechanism of polydipsia and polyuria accompanying DOCA administration has not been described, it was suggested forty years ago by Ragan *et al.* (1940) that the condition is primarily a thirst, perhaps related to the maintained increase in extracellular sodium, and only secondarily a polyuria. More recently Berl *et al.* (1977) presented evidence suggesting that potassium depletion affects water homeostasis by both central and renal mechanisms. These authors concluded that potassium depletion stimulated thirst leading to a primary polydipsia which is initially responsible for the polyuria. Further potassium depletion then results in a derangement of maximal urinary concentration which is independent of the polyuria and polydipsia and most likely due to a direct renal effect of hypokalemia. In these studies polydipsia was evident 6 days after commencement of a potassium deficient diet. A significant increase in urine output and decrease in urine osmolality were not evident until 1 or 2 days later.

The current study was conducted to describe the diabetes insipidus-like syndrome as it occurs in the intact Yucatan miniature boar following DOCA implantation.

MATERIALS AND METHODS

Intact, adult Yucatan miniature boars ranging in age from 18 to 20 months (70–100 kg), were housed in 4 × 6 ft metabolic cages and observed for 2–4 weeks before undergoing surgical procedures. During this period metabolic data were obtained and the animals became adjusted to handling. Throughout the study all animals received a pre-measured quantity of pig chow meal (Zeigler Brothers, Gardners, PA) supplemented with sodium chloride to allow a sodium intake of 4.5 mEq/kg/day. Water was provided *ad libitum*.

At the end of the adjustment period, animals were instrumented with a chronic indwelling carotid artery catheter for blood sample collection. Following preanesthesia with ketamine (20 mg/kg), the pigs were maintained at a surgical plane with a mixture of halothane, nitrous oxide and oxygen. The tip of the catheter was advanced into the thoracic descending aorta.

When 10 days of stable baseline measurements had been obtained, silicone (control) or DOCA-impregnated silicone (experimental) rubber strips were placed subcutaneously in the left or right flank under thiamylal (Surital, intra-arterial) anesthesia. Implants were prepared by mixing DOCA (Sigma Chemical) and rubber (Dow Corning) in a ratio of approximately 1:2. Total dosage was 100 mg DOCA/kg

body weight. All measurements were made between 9:00 a.m. and noon daily during the pre-implant observation period and for three weeks following DOCA implantation, and blood samples were obtained in the conscious, standing, unrestrained animal.

Serum sodium and potassium were determined with a Beckman Klina Flame flame photometer, and urine osmolality with an Advanced Instruments Digimatic osmometer. All values are reported as ±SEM. One-tailed, unpaired Student's t-tests were used to compare all data between experimental and control groups for one week before and one week after implantation to determine the day on which experimental values became significantly different from control. Differences were considered significant if $P < 0.004$. This level was chosen to reduce the possibility of Type I error since multiple tests were used (Glantz, 1980).

RESULTS

Water intake was significantly different from control by five days following implantation, having increased from approximately 31 to 40 ± 5 ml/kg/day (Fig. 1). By day +20, the water intake in the experimental group had increased to 55 ± 7 ml/kg/day. Intake in the control group remained relatively constant throughout the period of observation. Urine output was not different between the two groups prior to implantation (Fig. 2), but by day +7 the experimental group was significantly different from control ($P < 0.004$). As with water intake, by the end of the study period there was an approximate 2-fold increase from an average of 18 to 39.7 ± 7 ml/kg/day. Concomitant with the 2-fold increase in water turnover was a 2-fold decrease in urine osmolality which was significantly different from control by day +8 (Fig. 2).

Serum sodium was significantly elevated 48 and 72 hr after implantation, and again on day +7 (Fig. 3). It then remained significantly elevated for the remainder of the study. Twenty-four hours following DOCA administration serum potassium had de-

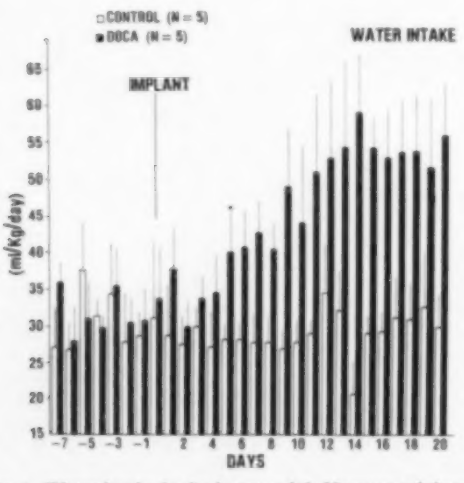

Fig. 1. Water intake in the intact, adult Yucatan miniature boar following implantation of desoxycorticosterone-acetate (DOCA). Water intake was significantly greater than control 5 days after implantation. Asterisk (*) indicates significance at $P < 0.004$ or less.

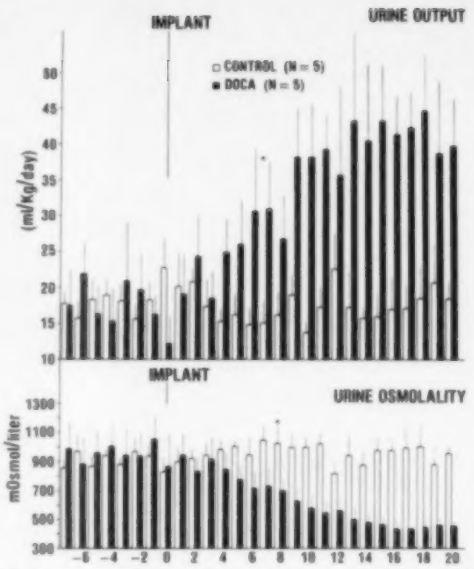

Fig. 2. Urine output and osmolality in the intact, adult Yucatan miniature boar following implantation of desoxycorticosterone-acetate (DOCA). Urine output was significantly greater than control 7 days after implantation and urine osmolality was significantly less than control by 8 days after implantation. Asterisk (*) indicates significance at $P < 0.004$ or less.

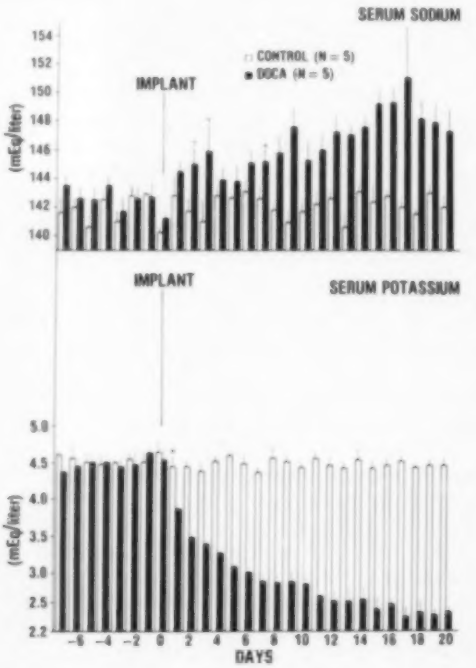

Fig. 3. Serum sodium and serum potassium in the intact, adult Yucatan miniature boar following implantation of desoxycorticosterone-acetate (DOCA). Serum sodium was significantly greater than control at 2, 3 and 7 days after implantation. Serum potassium was significantly less than control 24 hr after implantation. Asterisk (*) indicates significance at $P < 0.004$ or less.

creased from a preimplant level of 4.5 mEq/l (range 4.37–4.63) to 3.86 ± 0.09 mEq/l and was significantly different from control ($P < 0.004$) (Fig. 3). Potassium continued to decrease to 2.46 ± 0.06 by the end of the observation period.

DISCUSSION

Daily injections of 25 mg DOCA in the dog have resulted in increases in water intake from 500 to 3500 ml/day after 4 weeks (Ferribee *et al.*, 1941) or 2.5 to 4.0 l after several weeks (Kuhlman *et al.*, 1939). In the monkey, 25 mg DOCA resulted in a polydipsia that was evident 10 days after the start of the treatment (Knobil *et al.*, 1953). Smaller dosages in the dog (5 and 10 mg daily) have also produced polyuria and polydipsia (Moehlig and Jaffe, 1942). Injections in the rat ranging from 0.5 to 10 mg daily induced an increased water turnover (Selye *et al.*, 1943; Selye and Bassett, 1940; Rice and Richter, 1943), and chicks became polydipsic following the injection of 1–4 mg daily (Stamler *et al.*, 1951).

No studies of polyuria and polydipsia have been reported in these species following the implantation of DOCA, and the above studies do not describe the development of polyuria and polydipsia following DOCA administration. Mulinos *et al.* (1942), in studies with dogs, believed that the polydipsia appeared before the polyuria. Winter and Ingram (1943), also in the dog, disagreed, reporting that the polyuria and polydipsia seemed to keep pace with one another. Ragan *et al.* (1940) concluded that the condition they described was primarily a thirst since pituitrin was relatively ineffective in their animals and fluid restriction did not cause dehydration. Similar findings have also been reported by others (Moehlig and Jaffee, 1942; Mulinos *et al.*, 1942). In the present studies an increase in water intake preceded an increase in urine output by 24–48 hr, suggesting that the condition in the pig is initially a thirst, in agreement with the results previously cited in the dog. Additionally, although pitressin has not been employed in our studies, water restriction does not lead to dehydration (unpublished observation).

Serum potassium in the pigs was significantly decreased within 24 hr after DOCA implantation, and had decreased to approximately 2.9 mEq/l by the end of the first week. At this time a significant hypernatremia had developed which persisted throughout the remainder of the study. The hypokalemia which preceded an increased water turnover, and subsequent hypernatremia which coincided with it, could have resulted in cellular dehydration of central osmoreceptors (Fitzsimons, 1976) or stimulated postulated central sodium receptors (McKinley *et al.*, 1974), thereby causing thirst. Additionally, it has been shown that nephrectomy, which removes renal renin, results in an increase in cerebrospinal fluid angiotensin (Saad *et al.*, 1975). A reciprocal relationship between blood-borne and cerebrospinal fluid angiotensin II has been postulated (Fitzsimons, 1976). In fact, a recent study by Basso *et al.* (1981) has demonstrated an increase in isorenin activity in the brain following DOCA administration in the rat, and previous studies in the pig have shown that plasma renin activity decreases dramatically within 1 week following DOCA implantation (Grekin *et al.*, 1980; Terris and Simmonds, 1979). If such a reciprocal relationship does exist, there may also have been a central stimulation of thirst by an increased central angiotensin II during this period which contributed to the developing polydipsia.

Renal lesions have been seen in the collecting system of the rat accompanying potassium depletion, which are established by the end of the first week and fully developed at 2 weeks (Oliver *et al.*, 1957). An inability to concentrate the urine accompanies these lesions (Hollander *et al.*, 1957). It has also been suggested that potassium deficiency may interfere with the ability of the kidney to respond to antidiuretic hormone (Dousa, 1974).

The early development of hypokalemia, hypernatremia, and suppression of peripheral plasma renin activity seen in the pigs, affecting central osmoreceptors or sodium receptors, and/or increasing central angiotensin levels, may have initially been responsible for the polydipsia and resultant polyuria observed in this model. Insensitivity of the kidney to circulating antidiuretic hormone and hypokalemic renal lesions may then have potentiated the polyuria and polydipsia. This hypothesis will require further study.

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CONCENTRATIONS AND ACTIVITY COEFFICIENTS OF Na^+, K^+ AND Cl^- IN *APLYSIA CALIFORNICA* ENTEROCYTES

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Abstract—1. Tissue water averaged 76% of tissue weight in epithelial sheets of *Aplysia californica* intestine.
2. Extracellular water averaged 19.6% of total intestinal water.
3. Intracellular Cl^- concentrations averaged 110 mM with most of Cl^- being bound or sequestered.
4. Intracellular Na^+ concentrations were 34.2 mM with approximately 30% bound or sequestered.
5. Intracellular K^+ concentrations were not bound and averaged 532 mM.
6. Valinomycin lowered intracellular K^+ concentrations dramatically.

INTRODUCTION

In the absence of muscosally-added organic solutes, small transepithelial potential differences (ψ_{ms}) can normally be observed across vertebrate intestine, the serosal aspect being positive relative to the mucosal aspect (Baillien and Schoffeniels, 1961; Clarkson *et al.*, 1961). In fact, the short-circuit current (SCC) in these preparations has been shown to consist of either (a) active Na^+ transport from mucosa to serosa; (b) active Na^+ and Cl^- transport from mucosa to serosa (Barry *et al.*, 1965). Subsequent addition of an actively transported sugar or amino acid to the mucosal bathing medium of vertebrate intestine instantaneously enhanced both ψ_{ms} and SCC (Schultz and Curran, 1970). Enhanced Na^+ absorption accounted for these observed electrical increases (Quay and Armstrong, 1969).

Several observations indicate that electrolyte transport in invertebrate intestine differs from that in vertebrate intestine. For example, invertebrate intestine bathed in a Na^+-containing solution exhibits an electrical polarity such that the serosal aspect is negative relative to the mucosal aspect (Gerencser, 1978b; Lawrence and Mailman, 1967). Furthermore, the negative serosal ψ_{ms} could be hyperpolarized by metabolizable monosaccharides (Lawrence *et al.*, 1972). This observation is consistent with greater anion absorption rather than enhanced Na^+ absorption.

Recently, Gerencser (1978b) reported that the ψ_{ms} across the intestine of the mollusk, *Aplysia californica*, was serosa negative and the major portion of the SCC was carried by a net active Cl^- absorption while the remainder of the SCC was wholly or predominantly a net Na^+ absorptive current. Additionally, Gerencser (1978a) reported that addition of actively transported sugars and amino acids (Gerencser, 1981) to the mucosal bathing solution hyperpolarized ψ_{ms} and increased SCC across the *Aplysia* intestine. The major portion of the enhanced SCC in the presence of D-glucose (Gerencser, 1978a) or glycine (Gerencser, 1981) was carried by an enhanced net Cl^- absorption while the minor portion of the SCC was wholly or predominantly an enhanced Na^+ absorption (Gerencser, 1978a).

It is not known whether the differences between the intestine of the invertebrate *Aplysia* and vertebrate intestine reflect different mechanisms of transepithelial ion transport. To understand better the transfer of ions (Na^+, K^+, Cl^-) across the epithelial cell membranes, a knowledge of the intracellular ionic pools is essential. To the extent that Na^+, K^+, and Cl^- are transported through the epithelial cells, such active transport pools must exist. Ideally, a knowledge of the size and location of such pools within the transporting cells as well as the electrochemical potentials of these ions in their respective pools is essential to our understanding of their transepithelial transport.

Therefore, in the present study, measurements were made of extracellular water, total water and concentrations of Na^+, K^+ and Cl^- in the enterocytes of *Aplysia californica* intestine as well as calculations of the activity coefficients for these three ions were made.

MATERIALS AND METHODS

Mollusk

Sea hares, adult *Aplysia californica*, were obtained from Pacific Bio-Marine, Venice, California, and were maintained at 25°C in circulating filtered seawater.

Incubation media for intestinal tissue

The formula for the standard seawater Ringer's solution used was: NaCl, 0.462 M; $MgSO_4$, $7H_2O$, 0.00243 M; KCl, 0.0121 M; $NaHCO_3$, 0.00238 M; $MgCl_2$, $6H_2O$, 0.00983 M; $CaCl_2$, 0.01135 M. The total osmolality of the bathing medium was approximately 1000 mOsm/l and the final pH was 7.8 at 25°C. The activity coefficient of the seawater medium was calculated as 0.66 (Conway, 1952).

Intracellular Na^+ and K^+ concentrations were determined specifically in cells lining the villi of *Aplysia* intestine by the following method (White, 1976). Segments of intestine were cut open and pinned to paraffin in the bottom of a Lucite chamber containing continuously oxygenated sea-

Table 1.

	Total tissue water (%)	Extracellular water (%)	Intracellular water (%)
Before 9 μM valinomycin	76.2 ± 4.3 (5)	19.6 ± 3.1 (5)	84.8 ± 7.3 (5)
After 9 μM valinomycin	75.8 ± 5.6 (5)	21.3 ± 2.6 (5)	85.6 ± 8.1 (5)
Probability	NS	NS	NS

Values are means ± SEM. Numbers in parentheses are numbers of observations. Total tissue water is relative to tissue weight while extracellular water is relative to total tissue water. Intracellular water is calculated as the difference between total tissue water and extracellular water.

water. With a dissecting microscope and fine scissors, strips of villous tissue were cut from the intestine. These were incubated for 90 min in oxygenated seawater containing 1 mM unlabeled inulin. Some of the segments were exposed to ^{14}C-inulin (New England Nuclear) for the last 60 min to assess extracellular space. All segments were filtered by suction onto filter paper, blotted, and weighed in aluminum boats previously boiled in nitric acid. Villous segments exposed to ^{14}C-inulin were digested in 3 ml of 0.1 N HNO_3 for 48 hr with continuous shaking. Duplicate 1 ml aliquots were counted for radioactivity along with duplicate 0.1 ml aliquots of a 1:40 dilution (0.1 N HNO_3) of incubation solution.

The samples were counted in a three-channel Beckman LS-330 liquid scintillation counter. Two additional groups of villous segments were oven-dried at 100°C for 40 hr to obtain dry tissue weight. One group was solubilized in 25 ml 0.1 N HNO_3 and, after 48 hr of continuous shaking, analyzed for Na^+, K^+ and Cl^- with a Perkin–Elmer model 303 atomic absorption spectrophotometer.

All data are reported as means ± SEM. Differences between means were analyzed statistically using Student's t-test with a $P < 0.05$ used as the statistical significant difference criterion.

RESULTS

As seen in Table 1, in a normal NaCl seawater medium, the mean total water to epithelial tissue wet weight ratio averaged 76.2%. The tissue wet weight was comprised of a scraped epithelium devoid of muscle tissue. Extracellular water averaged 15.2% of the tissue wet weight whereas extracellular water averaged 19.6% of the total water of the epithelial tissue. By difference, therefore, intracellular water averaged 84.8% of tissue wet weight and intracellular water averaged 80.4% of total tissue water. Valinomycin (9 μM) had no significant effect on epithelial tissue wet weight, total water, intracellular water or extracellular water.

In order to determine the physico-chemical state of Na^+, K^+ and Cl^- in the *Aplysia* enterocyte, villi were surgically removed from the remaining mucosa and analyzed for Na^+, K^+ and Cl^-. Also, in the present study, the effect of 9 μM valinomycin on intracellular K^+ concentrations (C^i_K) was studied. As shown in Table 2 intracellular concentrations of Na^+ (C^i_{Na}), C^i_K and intracellular concentrations of Cl^- (C^i_{Cl}) averaged 34.2 ± 3.5, 532 ± 47 and 109.6 ± 4.3 meq/kg cell water, respectively. After valinomycin addition to the bathing medium there was a significant decrease in C^i_K ($P < 0.05$). That a significant amount of intracellular Na^+ and Cl^- is bound or compartmentalized is evident from the ratios a^i_{Na}/C^i_{Na} and a^i_{Cl}/C^i_{Cl}, which are equivalent to the activity coefficients (γ^i_{Na}, γ^i_{Cl}) of these ions. However, virtually no K^+ was found to be bound or compartmentalized (Table 2). The activity values used for calculating the activity coefficients of Na^+, K^+ and Cl^- were obtained from previous studies done under identical conditions (Gerencser and White, 1980; Gerencser, 1983).

DISCUSSION

Intracellular Na^+, K^+ and Cl^- concentrations were obtained from the villous epithelial cells of *Aplysia californica* intestine, as were the intracellular Na^+, K^+ and Cl^- activity measurements (Gerencser and White, 1980; Gerencser, 1983). This insures reasonably valid activity coefficients ($\gamma^i_K = a^i_x/C^i_x$) for the three ions when considering the heterogeneity of the intestine relative to villous, intervillous and crypt cell regions.

The data in Table 2 indicate that a large fraction of the Na^+ associated with the absorptive cells is not available to the Na^+-microelectrode since the experimental activity coefficients, a^i_{Na}/C^i_{Na}, is much lower than the activity coefficient for Na^+ in the bath (0.66). This is in conformity with the results previously reported for *Aplysia* intestine (Gerencser, 1983), bullfrog intestine maintained in sodium sulfate media (Armstrong *et al.*, 1975) or in sodium chloride media (Armstrong *et al.*, 1979a) and with the data reported for other species (Lev and Armstrong, 1975; Walker and Brown, 1977).

The C^i_{Cl} found in this investigation gave an γ^i_{Cl} of 16.7%. This is considerably lower than the value (0.66) predicted, on the basis of a free solution model

Table 2. Intracellular Na^+, K^+ and Cl^- concentrations and activity coefficients

	C^i_K (meq/kg cell water)	γ^i_K	C^i_{Na} (meq/kg cell water)	γ^i_{Na}	C^i_{Cl} (meq/kg cell water)	γ^i_{Cl}	N
Before 9 μM valinomycin	532 ± 47 (10)	0.72	34.2 ± 3.5 (10)	0.50	109.6 ± 4.3 (10)	0.11	5
After 9 μM valinomycin	14.4 ± 1.2 (10)						

Values are means ± SEM. Numbers in parentheses are numbers of observations; N is number of animals.

for cytoplasmic Cl^- and could be interpreted as suggestive of some intracellular binding or compartmentation of Cl^-. This conclusion is in concert with those findings of γ^i_{Cl} in *Amphiuma* small intestine (White, 1977), rabbit ileum (Frizzell *et al.*, 1973) and bullfrog small intestine (Armstrong *et al.*, 1979b).

It appears that most or all intracellular K^+ is in free solution in the cytosol. This agrees with the activity coefficient for K^+ found in *Aplysia* intestine (Gerencser, 1983), bullfrog intestinal epithelial cells (Lee and Armstrong, 1972) and a great many more species for which a_K has been directly measured (Lev and Armstrong, 1975; Walker and Brown, 1977). On the other hand a K^+ activity coefficient of 0.28 was reported for *Amphiuma* intestine (White, 1976) and, similarly, low values for K^+ activity coefficient were described (Khuri, 1974) for *Necturus* (0.57) and rat proximal tubule cells (0.40) and rat distal tubule cells (0.34).

It, therefore, appears that C^i_K reflects a true transport pool for K^+; however, C^i_{Na} severely overestimates the transport pool for Na^+ because of the significant fraction of intracellular Na^+ that is bound. The Cl^- transport pool is approximately 17% of the C^i_{Cl} which creates steep electrochemical gradients for this anion across both mucosal and basolateral membranes of the *Aplysia* enterocytes. This provides both a large downhill electrochemical driving force for Cl^- from the mucosal solution, across the mucosal membrane, into the cytoplasmic aspect of the enterocyte and a large uphill electrochemical driving force for Cl^- from the intracellular aspect of the enterocyte across the basolateral membrane into the serosal solution.

The finding that valinomycin decreases C^i_K to an extremely low value relative to control suggests that K^+ is virtually the only ion sensed by the K^+-sensitive microelectrode for valinomycin is a specific ionophore for K^+ (Krasne *et al.*, 1971). The possibility does exist, however, that interfering cations can contribute to the K^+-electrode potential. However, in light of the valinomycin effect on C^i_K the maximum contribution to the K^+-electrode potential by interfering cations would constitute only a minute portion (negligible) of the total potential. Also, this observation indirectly confirms the notion that K^+ is the major ion contributing to intracellular osmolality in the *Aplysia* intestinal epithelial cells.

Assuming that the intracellular activity coefficient for K^+ is the same as that in the external medium (Lev and Armstrong, 1975), the intracellular K^+ concentration found in other intestinal preparations (Armstrong *et al.*, 1979a; Garcia-Diaz *et al.*, 1980; Lee and Armstrong, 1972) accounts for some 45 to 60% of the total intracellular osmolality. The same calculation shows that my estimate of a^i_K is equivalent to an intracellular K^+ concentration of *ca.* 532 mM; that is, K^+ accounts for 53% of the total intracellular osmolality, in good agreement with earlier reports by others (Armstrong *et al.*, 1979a; Garcia-Diaz *et al.*, 1980; Lee and Armstrong, 1975).

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COMPARATIVE STUDY OF THE EFFECT OF HYDROCORTISONE AND THYROXINE ON SUCKLING MOUSE SMALL INTESTINE IN ORGAN CULTURE

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Abstract—1. The influence of hydrocortisone (10^{-8}–10^{-5} M) and thyroxine (10^{-9}–10^{-6} M) on intestinal epithelial cell differentiation and proliferation have been studied using explants of suckling mouse jejunum maintained in serum-free organ culture.

2. Hydrocortisone induced the appearance of sucrase activity and increased trehalase, glucoamylase, lactase and alkaline phosphatase activities.

3. Thyroxine was completely ineffective at all the concentrations used.

4. None of these hormones affected the mitotic activity or the ^{3}H-thymidine incorporation into DNA.

5. These results demonstrate that hydrocortisone but not thyroxine acts directly on intestinal brush border membrane differentiation and that both hormones do not influence the proliferation of the epithelial cells during postnatal development.

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INTRODUCTION

During the postnatal development of rodent small intestine, the mucosa undergoes substantial modifications affecting its structural and functional characteristics. During the past years considerable attention has been focused on the hormonal control of the maturation of the brush border enzymes using intact, hypophysectomized, thyroidectomized and adrenalectomized rats and mice. These studies showed that glucocorticoids and thyroxine are necessary for the normal intestinal brush border membrane maturation and have been the subject of many recent reviews (Henning, 1981; Koldovsky, 1981; Moog, 1981, 1982). In addition insulin and epidermal growth factor (EGF) have also been implicated in this regulatory mechanism (Ménard *et al.*, 1981; Malo and Ménard, 1982). On the other hand, changes in cellular turnover which represent another important aspect of the intestinal maturation, have received much less consideration (Herbst and Sunshine, 1969; Yeh and Moog, 1978).

The maintenance *in vitro* of mucosal biopsies and intestinal explants from the small intestine of humans and experimental animals now permits the study of synthetic, secretory and proliferative functions of intestinal mucosa (Trier, 1980; Hugon *et al.*, 1982). Because organ culture removes the studied tissue from the interacting complexities of the intact organism, it permits a systematic approach to the study of the intrinsic and extrinsic regulators of intestinal epithelial maturation than is possible *in vivo*. This methodology made possible investigations of the ontogenic development of the intestine of the fetal rat, mouse and chick (De Ritis, 1975; Black and Moog, 1978; Calvert *et al.*, 1981). Few investigations have been devoted to the analysis of intestinal development *in vitro* using suckling intestine (Shields *et al.*, 1979; Kedinger *et al.*, 1980; Simon *et al.*, 1982). We recently reported the successful maintenance of suckling mouse small intestine in a serum-free medium (Malo *et al.*, 1983).

The aim of this investigation was to gain more information regarding the direct or indirect involvement of hormones and/or factors in the regulatory mechanisms which govern the differentiation and proliferation of intestinal epithelial cells during postnatal development. The present paper reports the effects of hydrocortisone and thyroxine on the differentiation of brush border enzyme activities (disaccharidases, glucoamylase, alkaline phosphatase) and on the proliferation of crypt cells (mitotic index, ^{3}H-thymidine incorporation into DNA).

MATERIALS AND METHODS

Animals

Swiss ICR mice were bred in our own animal quarters. Litters were reduced to eight on the second postnatal day and the experiments were performed on 8-day-old suckling mice.

Organ culture

After decapitation of the animals, a 3 cm segment of jejunum extending from the ligament of Treitz was removed, split longitudinally, washed in culture medium and cut into small explants (3 × 4 mm). In order to get a direct comparison of the effects of the studied hormone, five animals originating from the same litter were sacrificed for each experiment. Thirty intestinal explants from each mouse were randomly distributed in five Falcon organ culture dishes (Falcon Plastics, Los Angeles, California, USA) vertically disposed on a plate and the experimental groups of explants were determined horizontally, therefore permitting the direct comparison of the effects of four different dosages of hormone with a control culture on the same tissues. Each experiment was repeated at least three times.

The explants were cultured in serum-free Leibovitz L-15 medium according to the technique described earlier (Malo

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et al., 1983). After a 3 hr period of stabilization for the explants, the following hormones were added to the culture medium: (1) hydrocortisone (Collaborative Research Inc., Massachusetts, USA) dissolved in ethanol and added at a concentration ranging from 10^{-8} to 10^{-5} M per ml; (2) DL-thyroxine (Sigma) dissolved in 0.005 N NaOH and added at a concentration ranging from 10^{-9} to 10^{-6} M per ml. Intestinal explants were cultured for 24 and 48 hr. The medium was changed after 24 hr for cultures lasting 48 hr.

Morphological study

Explants were fixed either in Bouin's fluid or in 2.8% glutaraldehyde buffered with 0.1 M cacodylate for light and electron microscopic study. The explants fixed in Bouin's fluid were subsequently dehydrated and embedded in Tissue-Prep and the sections strained with PAS or HPS techniques. Counts of mitotic figures were made on ten thousand crypt cells selected from three explants for each time period, and the determinations repeated at least three times. The mitotic activity was expressed as the percentage of cells in mitosis over the total number of crypt cells counted. Finally the explants fixed in glutaraldehyde were rinsed for 90 min in 0.1 M cacodylate buffer, postfixed in 2% osmium tetroxide for 30 min, dehydrated and embedded in epon. One-micrometer sections were stained with toluidine blue and ultra-thin sections double stained with uranyl acetate and lead citrate.

Enzyme study

After 24 and 48 hr of culture, the explants were pooled and homogenized into ice-cold redistilled water, with the Sorvall omnimixer equipped with the microattachment device, for 2 min at full speed. Sucrase, lactase and trehalase activities were assayed using the method of Dahlqvist as modified by Lloyd and Whelan (1969). Glucoamylase and alkaline phosphatase were assayed by the methods of Schlegel-Haueter *et al.* (1972) and Eichholz (1967), respectively. Protein was estimated using the method of Lowry *et al.* (1951) with bovine serum albumin as standard. Glucoamylase activity was expressed as micromoles of glucose liberated per min, while all other brush border enzyme activities were expressed as micromoles of substrate hydrolyzed per min per gram of protein or per gram of tissue. These enzymatic activities were also measured in the correspondent culture media.

Deoxyribonucleic acid synthesis

The incorporation of ^{3}H-thymidine into DNA was determined after different time intervals by adding 2 μCi of ^{3}H-thymidine (73.6 Ci-mmol; New England Nuclear, Boston, Massachusetts, USA) per ml of culture medium. At the end of the incubation periods, DNA was extracted (Malo *et al.*, 1983) and using calf thymus DNA as standard, the DNA content was determined by the Burton procedure as modified by Giles and Myers (1965). The incorporation of ^{3}H-thymidine into DNA was determined by counting 0.5 ml of the DNA-containing filtrate in a Packard liquid scintillation system (Packard Instrument Co., Inc., Downers Grove, Illinois, USA). Deoxyribonucleic acid synthesis was expressed as disintegrations per min per microgram DNA. DNA.

Statistical analysis

Results were reported as the mean ± SEM and the statistical significance of the difference of the means was determined by Student's *t*-test.

RESULTS

Morphology

The overall crypt–villus morphology was well maintained during 48 hr of culture as reflected by the presence of tall villi covered with well-differentiated enterocytes and of well preserved undifferentiated crypt cells having mitotic activity. The addition of different dosages of hydrocortisone or thyroxine did not modify the morphology of the intestinal mucosa (Fig. 1).

Brush border enzymatic activities

Hydrocortisone. During 48 hr of culture, the protein contents of intestinal explants remained constant and the different concentrations of hydrocortisone

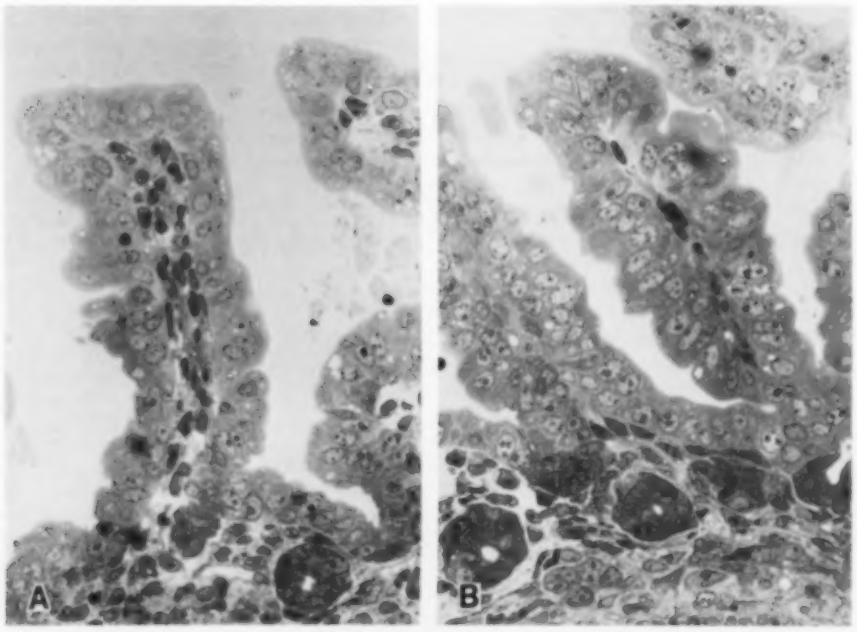

Fig. 1. Intestinal explants maintained in organ culture during 48 hr in presence of 10^{-8} M hydrocortisone (A) and 10^{-7} thyroxine (B). Villus and crypt architecture well preserved. Epon, toluidine blue. × 110.

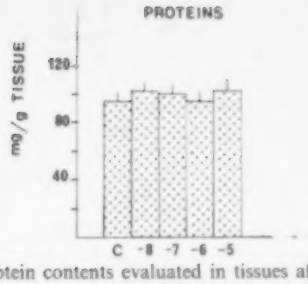

Fig. 2. Protein contents evaluated in tissues after 48 hr in control (C) and hydrocortisone-supplemented cultures (10^{-8}–10^{-5} M). Results expressed as the mean ± SEM.

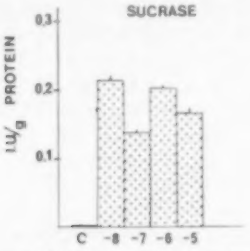

Fig. 3. Sucrase activity in intestinal explants after 48 hr of culture with and without different concentrations of hydrocortisone. Results expressed as the mean ± SEM.

(10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} M) did not significantly modify the protein concentrations (Fig. 2). The addition of hydrocortisone to the culture medium induced the appearance of sucrase activity in the intestinal explants as soon as 24 hr but this activity could not be detected in the medium even after 48 hr of culture (Fig. 3). Glucoamylase, trehalase, lactase and alkaline phosphatase activities were also increased in the intestinal explants by the addition of hydrocortisone to the medium, these increases representing respectivity 30, 27, 23 and 23% when compared to the control cultures. No significant variation of these enzymatic activities was recorded in the media (Figs 4, 5, 6 and 7).

Thyroxine. The addition of different concentrations of thyroxine (10^{-9}, 10^{-8}, 10^{-7} and 10^{-6} M) to the culture medium did not influence the protein contents of the explants (Fig. 8) and did not induce the appearance of sucrase activity in the tissue nor in the medium. Furthermore this hormone did not modify any of the other brush border membrane enzymatic activities in the explants or in the media (Fig. 9).

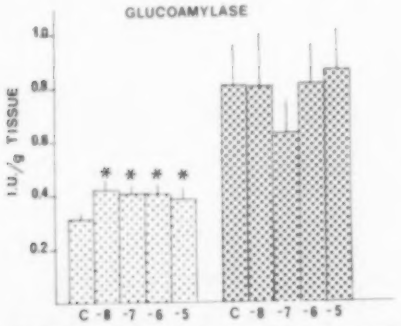

Fig. 4. Glucoamylase activity in tissues (cross-hatched bars) and corresponding media (dotted bars) after 48 hr of culture in control (C) and hydrocortisone-supplemented cultures (10^{-8} to 10^{-5} M). Results expressed as the mean ± SEM. *$P < 0.025$–0.0005.

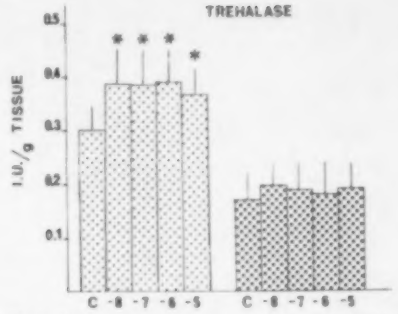

Fig. 5. Trehalase activity in tissues and corresponding media after 48 hr of culture. Symbols as in Fig. 4. *$P < 0.05$–0.025.

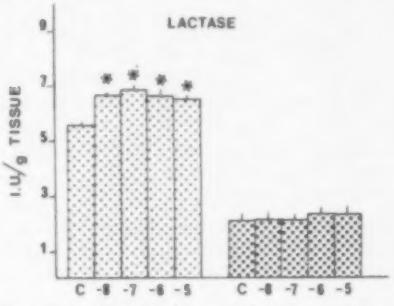

Fig. 6. Lactase activity in tissues and corresponding media after 48 hr of culture. Symbols as in Fig. 4. *$P < 0.01$–0.0005.

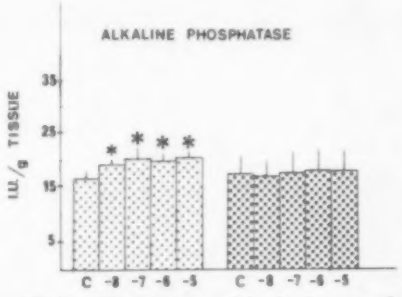

Fig. 7. Alkaline phosphatase activity in tissues and corresponding media after 48 hr of culture. Symbols as in Fig. 4. *$P < 0.01$–0.0005.

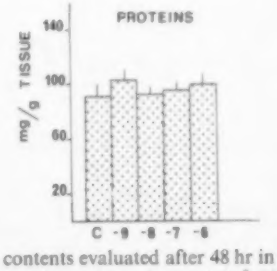

Fig. 8. Protein contents evaluated after 48 hr in control (C) and thyroxine-supplemented cultures (10^{-9}–10^{-6} M). Results expressed as the mean ± SEM.

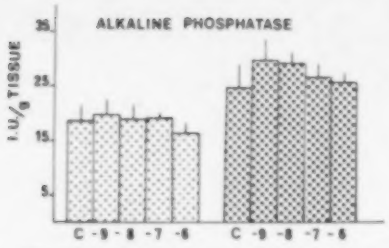

Fig. 9. Alkaline phosphatase activity in tissues (cross-hatched bars) and corresponding media (dotted bars) after 48 hr in control (C) and thyroxine-supplemented cultures (10^{-9}–10^{-6} M). Results expressed as the mean ± SEM.

Mitotic index and ^{3}H-thymidine incorporation into DNA

After 48 hr of culture, the mitotic index of the control explants slightly decreased as previously reported (Malo *et al.*, 1983). The addition of different concentration concentrations of hydrocortisone or thyroxine did not affect the mitotic activity (Table 1).

In order to determine an early action of these hormones on the DNA synthesis, ^{3}H-thymidine incorporation into DNA was evaluated after 12 and 24 hr of culture. Hydrocortisone and thyroxine did not influence DNA synthesis at any of the concentrations used after 12 hr (not shown) and 24 hr of culture (Table 2).

DISCUSSION

The modifications produced by hydrocortisone on brush border membrane activities clearly demonstrate in a serum-free medium, a direct effect of glucocorticoids on the induction of sucrase and on the stimulation of glucoamylase, trehalase, lactase and alkaline phosphatase activities of intestinal explants, without modifying the normal release of each enzyme in the medium (Malo *et al.*, 1983). When suckling rat intestine is maintained at room temperature in serum-free medium, hydrocortisone does not affect alkaline phosphatase during 24 hr (Shields *et al.*, 1979). This is also true for mouse small intestine sine it takes 48 hr to get a significant increase of alkaline phosphatase as well as of the other enzymes. The response *in vitro* of intestinal epithelial cells to the addition of hydrocortisone is in accordance to that reported *in vivo* (Doell and Kretchmer, 1964; Malo and Ménard, 1979a, 1979b, 1980; Henning, 1981; Koldovsky, 1981), even though the levels of stimulation are lower. This probably results from the temperature condition at which the culture was done. Raising the temperature up to 37°C does provide greater levels of stimulation after addition of hydrocortisone (unpublished data) but the morphology of the explants is greatly altered (Malo *et al.*, 1983).

Thyroxine has also been implicated in the regulatory mechanism of the maturation of intestinal brush border enzymes (Moog, 1981). In the present investigation thyroxine was completely ineffective regarding the induction of sucrase activity and the stimulation of the other brush border enzymes. This observation clearly confirms the recent report indicating that thyroxine plays no direct role in sucrase development when this hormone is administrated to adrenalectomized rat pups with serum corticosterone levels below 0.1 μg/dl (Martin and Henning, 1982). Since thyroxine at an optimal concentration of 10^{-8} M was able to stimulate alkaline phosphatase in cultured chick embryonic duodenum and since much of this activity was released into the medium (Black *et al.*, 1980), we studied this enzyme in the media after addition of different dosages of thyroxine. Unlike the chick, no variation of this activity as well as that of the other brush border enzymes was recorded. This different behaviour in response to thyroxine could be related either to age or to species differences.

This investigation has been extended to another important aspect of the postnatal maturation of the intestinal mucosa—the acceleration in the proliferation rate of the crypt cells. A recent study reported that thyroxine plays no direct role in sucrase development, but did not eliminate the possibility that thyroxine could indirectly control the sucrase development by affecting the rate of intestinal epithelial cell proliferation (Martin and Henning, 1982). The present observation shows that thyroxine as well as hydrocortisone does not influence the mitotic activity nor the ^{3}H-thymidine incorporation into DNA during 48 hr of culture. This lack of effect of thyroxine and hydrocortisone does not mean that the intestinal explants are unable to respond to a stimulator of cellular proliferation since insulin is able to increase these two parameters when added to culture medium (Arsenault and Ménard, 1982).

Table 1. Mitotic activity (‰) after 48 hr of culture*

	Control	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9} M
T (2)†	1.32 ± 0.13	ND	1.35 ± 0.12	1.30 ± 0.14	1.08 ± 0.08	1.23 ± 0.13
HC (3)†	1.70 ± 0.13	1.74 ± 0.10	1.68 ± 0.14	1.66 ± 0.13	1.71 ± 0.09	ND

*Mitotic activity expressed as percentage of cells in mitosis over total number of crypt cells counted.
†Number of experiments.
T: thyroxine; HC: hydrocortisone; ND: not determined.

Table 2. ^{3}H-thymidine incorporation into DNA after 24 hr of culture*

	Control	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9} M
T (4)†	476.3 ± 24.7	ND	520.1 ± 80.3	514.7 ± 85.8	473.2 ± 56.7	470.7 ± 67.3
HC (4)†	515.6 ± 57.0	551.2 ± 39.4	507.5 ± 35.4	501.5 ± 59.7	480.6 ± 36.6	ND

*^{3}H-thymidine was added to the different culture media for the last 3 hr of culture (21–24 hr) and the DNA synthesis was expressed as disintegrations per min per microgram DNA.
†Number of experiments.
T: thyroxine; HC: hydrocortisone; ND: not determined.

In conclusion, hydrocortisone but not thyroxine acts directly on suckling mouse intestinal tissue inducing the differentiation of the brush border membrane but is unable, along with thyroxine, to influence the proliferation of the epithelial cells.

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ABSENCE OF RESPIRATORY ACCLIMATION TO DIURNALLY-CYCLING TEMPERATURE CONDITIONS IN RAINBOW TROUT

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Abstract—1. Oxygen consumption and several ventilatory and cardiac functions were measured in rainbow trout normoxically acclimated to constant temperature conditions (2°, 10°, 18°C) or to a diurnal sinewave temperature cycle (10 ± 4°C). The former groups were also exposed to a single ±4°C cycle.

2. No evidence of diurnal variation in respiratory activity was apparent, and this probably reflects the sampling schedule employed rather than absence of circadian rhythmicity.

3. Temperature related increases in oxygen consumption were closely correlated with increases in ventilatory rate, flow and stroke volume. The latter were associated with increases in pressure generation by buccal and opercular pumps.

4. Heart rate increased with temperature, and electrocardiogram records suggest that this may have been accompanied by increased cardiac output.

5. Enhanced oxygen utilization at higher temperature may stem from lamellar flow adjustments leading to increases in effective branchial area.

6. There was no evidence of acclimation to cycling temperature conditions *per se*.

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INTRODUCTION

The relationship between environmental temperature and the respiratory metabolism of fishes has been extensively documented (e.g. Fry and Hochachka, 1971; Fry, 1971; Alderdice, 1976). Although some consideration has been given to the effects of abrupt temperature changes, interest has largely centered on the consequences of relatively long-term acclimation to precisely controlled conditions. Neither circumstance is normally encountered in the freshwater environment. Many species are, however, exposed to reasonably regular temperature fluctuations as a consequence of vertical or horizontal feeding and other movements, or because of the thermal characteristics of their habitats.

Relatively little is known with respect to the effects of these more ecologically realistic temperature conditions. Duthie and Houlihan (1982) have, however, recently examined oxygen consumption in flounder, *Platichthys flesus*, acclimated to constant temperature conditions or to 24 hr modified square-wave cycles. Similarly, Vondracek *et al.*, (1982) have compared the respiratory metabolism of the Tahoe sucker, *Catostomus tahoenis*, maintained at constant temperature with that of animals exposed to a diurnal cycle mimicking conditions in their natural habitat. In neither study was there evidence of acclimation to cycling conditions. This is unexpected. Earlier studies have correlated exposure to regular temperature fluctuations with significant alterations in functions ranging from thermal tolerance to enzyme activities (Heath, 1963; Feldmeth *et al.*, 1974; Otto, 1974; Houston and Rupert, 1976; Koss and Houston, 1981; Beaumont *et al.*, 1981; Houston and Koss, 1982; Threader and Houston, 1983).

Accordingly, in the present study we have examined oxygen consumption and selected ventilatory and cardiac activities in rainbow trout, *Salmo gairdneri*. Consideration has been given to the effects of relatively long-term acclimation to constant temperature conditions (2°, 10°, 18°C) and to a diurnal sinewave temperature cycle (10 ± 4°C). In addition, the responses of trout previously acclimated to constant temperatures to an initial ±4°C cycle have also been evaluated.

MATERIALS AND METHODS

Origin and maintenance of animals

Yearling rainbow trout ranging in weight from 326 to 429 g (mean ± 1 SD, 404.2 ± 36.9 g) were obtained from a commercial supplier (Goossens Trout Farm, Otterville, Ontario) and held in 500 l. fiberglass recirculating tanks. Supplementary water inflows provided 2–3 volume replacements daily. Each tank was equipped with a light-tight hood, and a 12 hr light–12 hr darkness photoperiod regime initiated at 08:30 hr was maintained throughout acclimation and test periods. The dechlorinated St Catharines city water used varied little in total hardness (140–145 mg l^{-1}, as $CaCO_3$), total alkalinity (95–100 mg l^{-1}, as $CaCO_3$) or pH (7.5–8.0) during the course of the study. Dissolved oxygen levels were inversely related to temperature, but exceeded 80% saturation. Animals were fed daily (09:00, 13:00 hr) to satiation, and excess food and faeces removed immediately thereafter.

Temperature conditions

Upon arrival in the laboratory, trout were weighed and distributed among the acclimation tanks with no significant differences in mean weights and comparable weight ranges. Following resumption of normal feeding temperatures were adjusted by approximately 1°C day^{-1} until the desired levels (2°, 10°, 18°C) were attained. In the instance of the group

to be exposed to diurnally-cycling temperature conditions a constant temperature of 10°C was first established. Cycle amplitude was then increased by ~0.5°C day^{-1} to 10 ± 4°C.

In the constant temperature acclimation tanks recirculating refrigeration–aeration units were operated against 1000 W stainless steel immersion heaters using locally designed and constructed duty operated regulators in conjunction with platinum resistance thermistors. Temperatures were continuously recorded and verified by US Bureau of Standards certified thermometer. Maximum excursions from cited values did not exceed 0.15°C. Establishment of cycling temperature conditions required amplification of heating and cooling capacities. The low-frequency sinewave generator used to drive the thermoregulator was therefore arranged to bring a second 1000 W heater into operation during the heating cycle. A commercial freezer was modified to provide continuous chilling of an antifreeze bath. On commencement of the cooling cycle a pump activated by the regulator delivered this on a demand basis to stainless steel coils immersed in the tank. Cycle periods were accurate to 7 min day^{-1}, and were corrected daily at 15:30 hr. The average maximum deviation of successive cycle peak and minimum temperatures was 0.18°C; that of cycle midpoints 0.08°C. Animals were acclimated for at least four weeks before use.

Specimen preparation

Individual animals previously starved for 24 hr were anesthetized at acclimation temperature in freshly prepared tricaine methane sulphonate (Ayerst "Finquel", 100 mg l^{-1}). On attainment of a surgical plane of anesthesia the specimen was transferred to an operating assembly which provided for continuous irrigation of the gills with dilute anesthetic solution (Houston *et al.*, 1969). Three subdermal pin electrodes were positioned for electrocardiogram recording and anchored with sutures (Houston *et al.*, 1973). A stainless steel trochar was employed as a guide through which a PE-160 catheter, heat-flared at one end, was placed in the roof of the buccal cavity. This was externally secured with a short length of flanged PE-240 tubing clipped to the catheter. Cleitheral catheters were positioned in the same fashion, and all catheters and electrode cables passed through a short length of tubing sutured anterior to the dorsal fin. Including anesthetization the duration of each preparation was 25–30 min.

The specimen was then transferred to a plexiglass restraining cradle, loosely secured to this with sutures through the dorsal and caudal fins and inserted into the respirometer chamber. These maneuvers were carried out with the animal immersed in water to facilitate recovery and minimize the stress associated with air exposure. Recovery in the respirometer, as evidenced by resumption of regular ventilatory activity, was normally evident within 15–20 min.

Determination of respiratory activities

The respirometer used was a modified form (Houston *et al.*, 1973) of the constant pressure head system described by Beamish and Mookerjii (1964), and incorporated specimen restraint features based on those used by Holmes and Stainer (1966) in renal function studies. The upper component containing the respirometer chamber was insulated and shielded to minimize specimen disturbance. The lower reservoir was provided with main and supplementary heating and cooling systems as previously described, and equipped with aeration and filtration units. Flow through the respirometer chamber was regulated by a micrometer valve-equipped Gilson calibrated flowmeter with a rated accuracy of ±2%. Chamber flow was maintained at 250 ml min^{-1}, and checked at each sampling period by timed filling of a volumetric flask. Oxygen consumption was estimated from values for chamber flow, and input and output water oxygen content; the latter being determined by means of the phenylarsene oxide modification of the Winkler procedure. Since quantitative determinations of activity levels are not possible in a system of this kind, oxygen consumption rates were considered representative of routine levels of metabolism.

Electrocardiograms were recorded on a Grass model 7B polygraph. Heart rates, QRS amplitudes and P–Q, Q–S and S–T intervals were estimated from at least three groups of 25 consequentive recordings from each animal. The intervals measured correspond to the period between atrial and ventricular contractions, the duration of ventricular contraction and the time required for ventricular repolarization. QRS amplitude values are suggestive of force of ventricular contraction. These, however, tended to be variable; small differences in electrode placement commonly leading to relatively large differences in recorded amplitudes.

Buccal and opercular pressures were recorded using Statham P23BC pressure transducers linked to Grass 7P/B low-level DC preamplifers. Ventilatory pump performance was estimated in terms of mean maximum buccal and minimum opercular pressures using the records employed in evaluation of cardiac activity. These records were also analyzed for evidence of cardiac and ventilatory synchrony, but yielded no evidence of sustained, consistent patterns.

Ventilatory flow was estimated by the Fick procedure, and stroke volumes calculated from ventilatory flow and rate. Gross oxygen utilization was also determined (Randall, 1970). These are primarily of relative value since their calculation incorporates a critical assumption; that water sampled from the post-branchial chamber represents a fully mixed expiratory flow. Davis and Watters (1970) have demonstrated that catheter positioning is critical in this regard. Cleithral sites, however, lead to less variable results (J. C. Davis, personal communication; Houston *et al.*, 1973), and it may be noted that the values obtained in this study compare favourably with those reported for this species on the basis of a catheter-less system for flow determination (Cameron and Davis, 1970).

Schedule

All studies were conducted using the following schedule. Each specimen was prepared between 08:00 and 09:00 hr, and placed in the respirometer. The following morning at 09:00 hr catheters and electrodes were connected, and the recording system calibrated. The specimen was left undisturbed for a further 12 hr period. The total recovery period, 36 hr, is regarded as adequate in terms of the parameters of interest (Houston *et al.*, 1973). Recording was initiated at 20:00 hr, and continued at 6 hr intervals for 48 hr. Recordings were completed prior to photoperiod changes, and those made during dark periods were conducted under dim, red illumination to minimize specimen disturbance.

In the case of animals acclimated to constant temperature conditions determinations were initially carried out over a 24 hr period (0, 6, 12, 18, 24 hr) to evaluate possible diurnal variations in activity, and establish baseline temperature–activity relationships. Upon completion of this, a ±4°C sinewave cycle was initiated and additional observations made at 30 (minimum), 36 (midpoint), 42 (maximum) and 48 hr (midpoint). Animals previously acclimated to 2°C were exposed only to the heating cycle and, to ensure coincidence in time, this was initiated at 36 hr. These data provided a basis for comparison of responses to temperature change in animals experiencing a first cycle with those of trout previously acclimated to cycling temperature conditions for 4 weeks or more.

Statistical treatment

Sampling was continued until 10 complete 48 hr data sets were obtained for each temperature treatment. As no significant differences in group weights were evident (means ± 95% confidence intervals of the mean: 10 ± 4°C—423 ± 14 g, 2°C—391 ± 17 g, 10°C—408 ± 27 g,

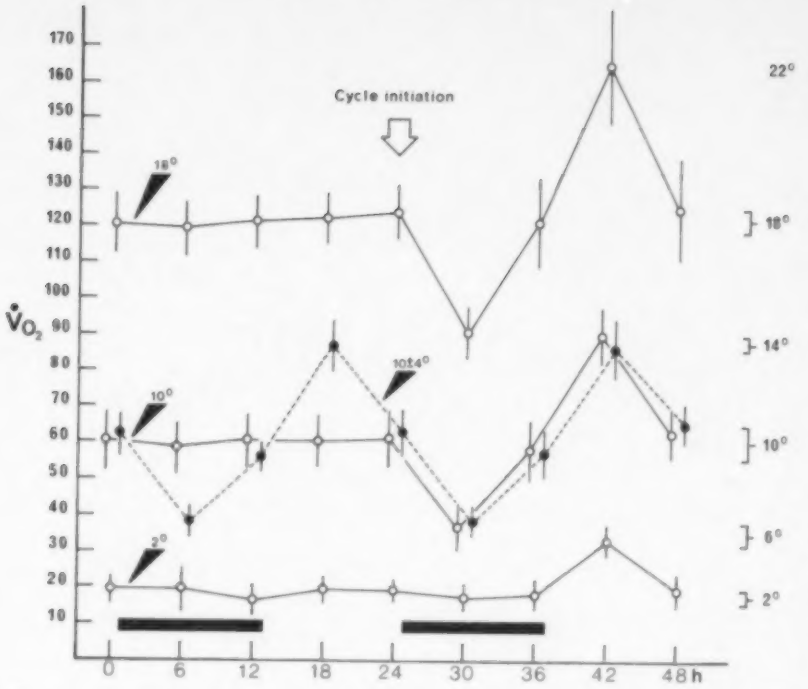

Fig. 1. Oxygen consumption (mg kg^{-1} hr^{-1}) as a function of time in rainbow trout exposed to constant (2°, 10°, 18°C) or diurnally-cycling (10 ± 4°C) temperature conditions, or to an initial temperature cycle. Observations reported as means ± 95% confidence intervals of the mean, $N = 10$.

18°C—415 ± 30 g) no attempt was made to correct values to a standard specimen weight. Group averages were expressed as means ± 95% confidence intervals of the mean for descriptive purposes. One-way analysis of variance, carried out following base 10 logarithmic data transformation, was used for group comparisons, and significance attributed to difference at the 0.05 level or better.

RESULTS

Oxygen consumption

Animals acclimated to constant temperatures exhibited no significant diurnal variations in oxygen consumption rates (Fig. 1; 0–24 hr). This was also true of other activities. Consequently, in subsequent figures these values have been treated as single samples. In the case of trout at 10 and 18°C each comprises the observations from 5 sampling periods (0, 6, 12, 18, 24 hr). For fish at 2°C, 7 data sets are included, i.e. 0–36 hr.

Mean oxygen consumption over the initial 24 hr period in animals acclimated to 10°C was almost identical to that observed in trout sampled at corresponding temperatures (0, 12, 24, 36, 48 hr) over the 10 ± 4°C cycle (10°C—60.1 ± 7.26 mg kg^{-1} hr^{-1}; 10 ± 4°C—59.7 ± 5.52 mg kg^{-1} hr^{-1}). Imposition of an initial ±4°C cycle on the former group did not lead to any significant differences between these animals and those acclimated to cycling conditions (Fig. 2). This was also true of other values obtained at comparable temperatures, regardless of the prior thermal experience of the animals. Thus, trout acclimated to 18°C and exposed to a 4°C decrease in temperature displayed oxygen consumption rates similar to those of cycled fish at 14°C, and 10°C animals raised to 14°C on the initial cycle. Similarly, animals previously acclimated to 2°C and exposed to 6°C were not significantly different from cycled trout at 6°C, or from specimens initially acclimated to 10°C and experiencing a decrease of 4°C.

Q_{10} values (Table 1) varied inversely with temperature, falling from 3.78–4.56 between 2° and 6°C to 1.99–2.21 over the 14° to 22°C range.

Ventilatory activity

Ventilatory rate, flow and stroke volume also exhibited no significant diurnal variation. All increased with increases in temperature, rising by 2.57, 5.50 and 2.36× respectively between 2° and 22°C (Fig. 3). Again there were no significant differences between treatment groups attributable to the nature of the acclimation regimes employed. Q_{10} values for ventilatory rate and flow declined with increase in temperature (Table 1). Both were substantially lower than corresponding values for oxygen consumption rates. Those for ventilatory rate were particularly low, ranging between 1.27 and 1.82.

In general, the same considerations applied to values for mean maximum buccal and opercular pressures as well (Fig. 4). In this instance, however, trout acclimated to 2°C exhibited significant ($P < 0.05$) differences from the other test groups when exposed to 6°C. Buccal pressures, for example, were above those of cycled fish at this temperature, and this was also true of animals acclimated to 10°C and shifted to 6°C. In addition, these animals were

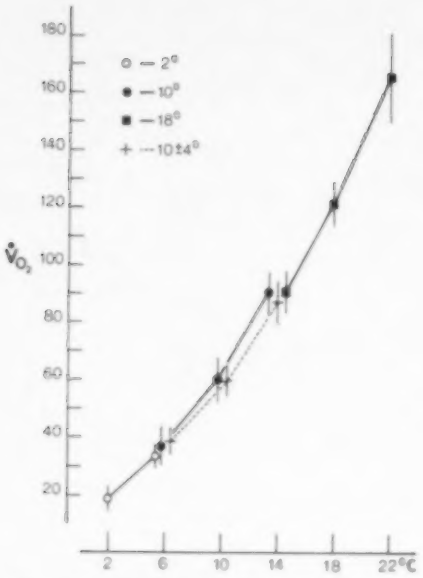

Fig. 2. Oxygen consumption (mg kg^{-1} hr^{-1}) as a function of temperature. Values reported as means ± 95% confidence intervals of the mean for all observations at the temperatures indicated.

characterized by (barely) significant differences between their opercular pressures and those of the group initially acclimated to 10°C. These data, as a whole, suggest that the buccal and opercular pumps contributed equally to the increase (~6.8×) in maximum pressure differential (mean maximum buccal pressure − mean minimum opercular pressure) generated during the ventilatory cycle over the temperature range considered. Values for animals acclimated to and tested at constant temperature, however, indicate that the opercular pump assumed increasing importance at higher temperatures under such circumstances.

Mean stroke volume proved to be closely correlated with maximum buccal–opercular pressure differential at all temperatures other than 2°C, i.e. mean stroke volume = 1.12 + 0.157 pressure differential ($r = 0.977$, $P < 0.01$). These variables were also positively and significantly correlated in cold-acclimated animals at 2 and 6°C. Stroke volumes were, however, some 25% below the values which would be anticipated from the foregoing relationship.

Finally, oxygen utilization rose sharply with temperature, increasing from 18.7 ± 3.8% at 2°C to 61.2 ± 6.3% at 22°C (Fig. 4). Much of this increase took, place between 2° and 14°C. Again, trout initially acclimated to 2°C differed from those on alternative thermal regimes when tested at 6°C. Utilization was significantly lower in these animals than in trout maintained on the 10 ± 4°C. No other significant differences were detected in the various group comparisons.

Cardiac activity

As was the case with oxygen consumption and ventilation, cardiac activity did not exhibit significant

Table 1. Mean Q_{10} values for oxygen consumption, ventilatory rate and flow and cardiac rate, 24–48 hr

Temperature regime	2°C		10°C			10 ± 4°C				18°C			
Temperature change	2→6	6→2	10→6	6→10	10→14	14→10	10→6	6→10	10→14	14→10	18→14	14→18	18→22	22→18
Oxygen consumption	4.56	−3.78	−3.45	3.12	2.98	−2.44	−3.35	2.61	2.91	−2.13	−2.21	2.09	2.17	−1.99
Ventilatory rate	1.53	−1.82	−1.58	1.44	1.61	−1.68	−1.81	1.70	1.81	−1.68	−1.52	1.52	1.47	−1.27
Ventilatory flow	3.06	−2.23	−2.35	2.21	2.42	−2.02	−2.57	2.07	2.57	−2.02	−1.97	2.05	2.41	−2.18
Cardiac rate	2.03	−2.11	−1.93	1.86	1.94	−1.97	−2.44	1.93	1.91	−1.54	−2.03	1.89	−0.96	−1.07

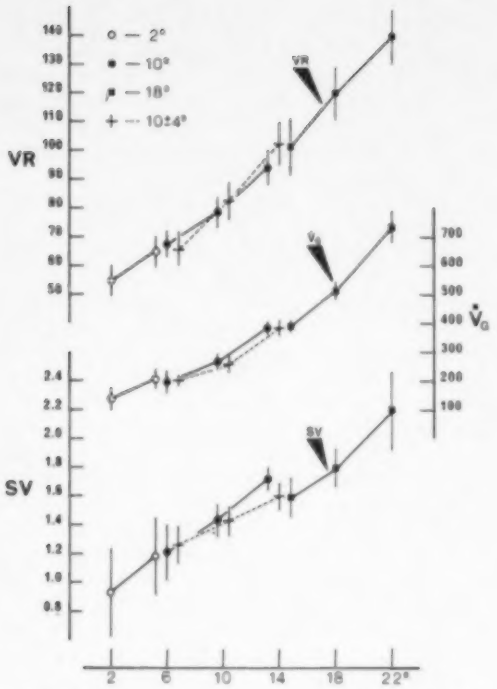

Fig. 3. Ventilatory rate (VR, cycles min^{-1}), flow ($\dot{V}_G$, $ml\ kg^{-1}\ min^{-1}$) and stroke volume (SV, ml) as a function of temperature. Values reported as mean ± 95% confidence intervals of the mean for all observations at the temperatures indicated.

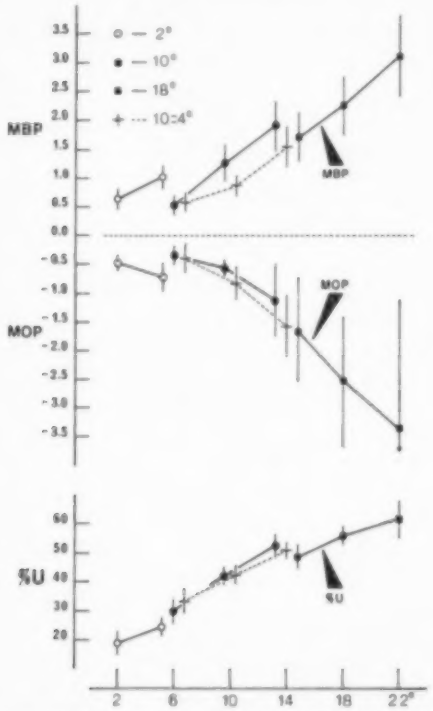

Fig. 4. Maximum buccal (MBP, mm Hg) and minimum opercular pressures (MOP, mm Hg) and oxygen utilization (%) as a function of temperature. Values reported as means ± 95% confidence intervals of the mean for all observations at the temperatures indicated.

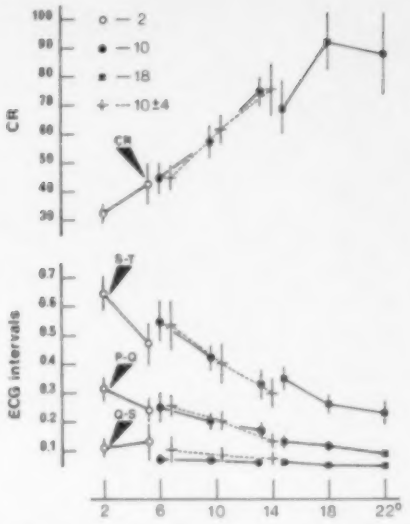

Fig. 5. Cardiac rate (CR, beats min^{-1} and S–T, P–Q and Q–S intervals (sec) as a function of temperature. Values reported as means ± 95% confidence intervals of the mean for all observations at the temperatures indicated.

diurnal variation. Similarly, while cardiac activity increased markedly with increase in temperature, there was no evidence that rates were differentially influenced by the thermal regime to which the animal had been exposed (Fig. 5). Maximum activity was observed at 18°C and, consistent with earlier observations on this species (Roberts, 1973; Heath, 1973), did not increase significantly at higher temperatures.

P–Q, Q–S and S–T intervals declined in what proved to be a negative logarithmic function of temperature (Fig. 5). None exhibited significant diurnal variation, and there was no evidence of acclimation to diurnally-cycling conditions. Finally, as anticipated, values for QRS amplitude were highly variable in nature. Nevertheless, in the mean sense at least, amplitude tended to increase at higher temperature.

DISCUSSION

Values for standard or routine oxygen consumption rates and related ventilatory and cardiac activities in rainbow trout held at constant temperatures have been reported by Holeton and Randall (1967), Stevens and Randall (1967), Davis and Cameron (1970), Hughes and Roberts (1970), Davis (1971), Holeton (1971), Davis and Randall (1973), Roberts (1973) and Kiceniuk and Jones (1977) among others. Some variability is evident in these reports, and presumably reflects differences in the preparative and recording methods employed. Nevertheless, the values obtained for oxygen consumption in the present study were in reasonably good agreement with these, and this was true as well of the other activities and parameters considered.

Absence of circadian variation in respiratory activity

Endogenous physiological rhythmicities are common in fishes (Schwassmann, 1971) and, in the in-

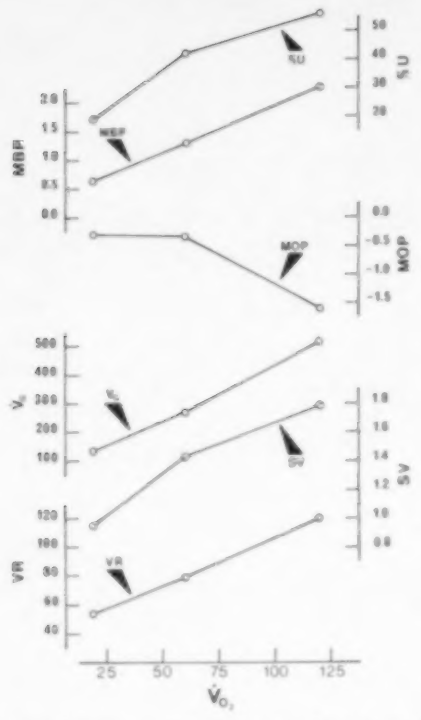

Fig. 6. Ventilatory rate (VR, cycles min^{-1}), stroke volume (SV, ml) ventilatory flow (V_G, ml kg^{-1} min^{-1}), maximum buccal (MBP, mm Hg) and minimum opercular pressure (MOP, mm Hg) and utlization (% *U*) as a function of oxygen consumption in animals acclimated to constant temperature conditions. Values reported as means for the initial 24 hr period of observation.

stance of respiratory activity, have been known for many years (e.g. Clausen, 1936). Their absence, particularly in animals acclimated to constant temperature and photoperiod conditions, was unexpected. Nevertheless, neither group means nor analysis of records from individual animals provided compelling evidence of circadian variation. Schwassmann (1971) has suggested that failure to detect such rhythms commonly stems from lack of sensitivity in the recording systems used. This argument would seem applicable to oxygen consumption determinations and to the indirect measurements made in the study (e.g. oxygen utilization, ventilatory flow, stroke volume), but less so to determinations of cardiac and ventilatory rates. It is more probable that failure to detect circadian variation reflects the sampling schedule employed. Unless samples are taken at intervals coincident or near-coincident with circadian maxima and minima obvious differences would not be apparent. For example, Clausen (1936) found oxygen consumption maxima in *Micropterus salmoides* between ~05:00 and 07:00 and ~19:00 and 22:00 hr; minima at 01:00 to 02:00 and ~09:00 to 12:00 hr. The schedule employed in the present study would have detected only the latter. It seems reasonable to speculate then, that any manifestations of circadian activity rhythms were probably between the sampling times selected.

Response to increased temperature

The general nature of systemic response to temperature-induced increases in the oxygen requirements of animals acclimated to constant temperature is suggested in Figs 6 and 7. These summarize, as a function of mean oxygen consumption, means for the various activities considered. Increased oxygen consumption was well correlated with increases in ventilatory rate and stroke volume. Ventilatory rate increased by 122% and stroke volume by almost 90% between 2° and 18°C. Ventilatory flow, a product function of these variables, was increased by a factor of 3.8. Increases in stroke volume were associated with major increases in buccal and opercular pump pressure generation. Increased ventilatory flow commonly leads to the shunting of an increased proportion of total flow through the non-exchange volumes of the gill system (Randall, 1970). Because of this, utilization normally falls as ventilatory flow increases. In this instance, however, flow and utilization rose at higher temperatures. This points to the intervention of some factor or factors enhancing utilization. The most obvious of these involves amplification of effective branchial exchange area; a crucial feature of response to both exercise related increases in metabolic rate and reduction in oxygen availability (Taylor *et al.*, 1968). Davis (1972) and Cameron (1974) have demonstrated that area increases can be accomplished by increasing the number of lamellar perfused. Accordingly, it is reasonable to speculate that a response of this kind may have contributed to the observed increase in utilization.

Heart rate was also increased with increases in oxygen consumption, rising by 2.8× over the tem-

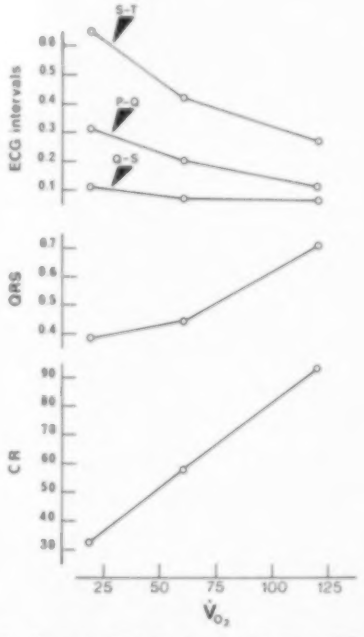

Fig. 7. Cardiac rate (CR, beats min^{-1}), mean QRS amplitude (QRS, mV) and S–T, P–Q and Q–S intervals (sec) as a function of oxygen consumption in animals acclimated to constant temperature conditions. Values reported as means for the initial 24 hr period of observation.

perature range considered. However, variations in P–Q and Q–S intervals suggest that both ventricular filling and contraction periods decreased at higher temperature, and this could lead to reduction in cardiac stroke volume. On the other hand, QRS amplitude increases under such circumstances, while S–T intervals fell. The first may be indicative of an increase in force of ventricular contraction. The latter points to an acceleration of ventricular repolarization following contraction. Consequently, stroke volume may not necessarily decline at higher temperatures. That of the lingcod, *Ophiodon elongatus*, for example, is little altered following temperature, increases which lead to a doubling in heart rate (Randall, 1968). Should this be the case cardiac output, and hence gill perfusion rate, would, like ventilatory flow, rise with increases in temperature and oxygen demand.

Oxygen uptake at the gills and delivery to the tissues is, of course, also a function of blood oxygen-carrying capacity and hemoglobin–oxygen affinity. Several studies on the rainbow trout have revealed only modest changes in both as a consequence of the acclimatory process (Cameron, 1971; Weber *et al.*, 1976; Houston, 1980; Nikinnma *et al.*, 1976). In this species, at least resolution of the temperature–oxygen demand problem apparently pivots upon systemic responses.

Cycling temperature conditions

Two principal observations concerning cycling temperature conditions have emerged from the present study. The first concerns the absence of any evidence indicative of acclimation to cycling conditions *per se*; the second lies in the absence of over- and undershooting responses to the initial cycle imposed on animals previously acclimated to constant temperatures. The first is entirely consistent with the earlier observations of Duthie and Houlihan (1982) and Vondracek *et al.* (1982) on oxygen consumption rates in flounder and sucker. Neither encountered significant differences between animals held on constant and fluctuating temperature regimes. Duthie and Houlihan (1982) did, however, observe overshoot and undershoot responses in flounder shifted from constant temperature conditions (5°, 15°C) to a 5–15°C square-wave cycle. In the instance of 15°C-acclimated animals this persisted for about two cycles. Shift of cold-acclimated animals to cycling conditions lead to oscillations requiring 5 or 6 cycles for stabilization. The principal difference between this, and the present study involves the nature of the cycles imposed on the animals, and may have some bearing on the discrepancy in results. Duthie and Houlihan (1982) employed a modified square wave cycle involving linear heating and cooling at rates of approximately 0.05°C min^{-1}, and involved substantially longer intervals at constant 5° than at constant 15°C. The rate of temperature change in the present sinewave cycle varied with time, diminishing as cycle peaks and minima were approached but averaging ~0.01°C min^{-1} over the entire cycle. If, as has been suggested, overshoot and undershoot responses to temperature changes are triggered by core–peripheral temperature differentials the substantially lower rates of temperature change involved in the present study may well have precluded their initiation.

Both the basis of this aparent absence of acclimatory response to cycling temperature conditions and its significance are presently unclear. The phenomenon stands, however, in distinct contrast to the effects which fluctuating temperatures have upon a wide range of physiological activities and capabilities in fishes. Exposure to regular temperature variation leads, for example, to increased heat tolerance and resistance in species as diverse as the rainbow and cutthroat trout (Heath, 1963; Threader and Houston, 1983), desert pupfish (Feldmeth *et al.*, 1974) and western mosquitofish (Otto, 1974). Several aspects of water-electrolyte regulation in goldfish are differentially influenced by exposure to constant and diurnally cycling temperatures (Houston and Koss, 1982). Total and mean erythrocytic hemoglobin concentrations, the abundancies of specific hemoglobin polymorphs and the cellular conditions in which these hemoglobins operate in the goldfish are also significantly affected (Houston and Rupert, 1976; Koss and Houston, 1981). This is true as well of red cell carbonic anhydrase activity and temperature sensitivity in this species (Beaumont *et al.*, 1981). Nevertheless, the three species so far examined have provided a generally consistent pattern in this respect.

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SEASONAL CHANGES OF FOOD AND WATER CONSUMPTION AND URINE PRODUCTION OF THE MARMOT, *MARMOTA FLAVIVENTRIS*

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Abstract—1. In early spring, food and water consumption and the excretion and clearances of urine and solutes reached maximal rates. Water consumption exceeded food intake and urine production and plasma osmolality was lowest.

2. Toward early and late summer, water intake decreased faster than food consumption and urine production. Urea excretion and clearances diminished with food consumption, while creatinine clearance decreased only slightly. Plasma osmolality increased.

3. The data are consistent with rehydration soon after hibernation is completed, followed by a period of weight gain and dehydration in preparation for the next prolonged period of hibernation.

INTRODUCTION

In a recent publication (Zatzman and South, 1981) we documented the seasonal nature of renal function and some plasma constituents of the yellow-bellied marmot (*M. flaviventris*). Although data of seasonal food intake and body weights of some hibernants are available (Pengelly and Fisher, 1963; Davis, 1976; Fall, 1971; Armtage *et al.*, 1976; Young and Sims, 1979; Hock, 1969), the literature contains no information of food and water consumption, urine production or urinary constituents of the *M. flaviventris* under laboratory conditions.

This report presents the data obtained from two studies of the yellow-bellied marmot: (1) weekly food and water consumption and bimonthly body weights of 18 animals (9 males and 9 females) and (2) weekly food and water consumption, electrolyte intake and excretion, urine production and constituents, bimonthly body weights and monthly determinations of plasma constituents, urea, creatinine, free water, and osmotic clearances in six animals (3 males and 3 females).

Of interest in these studies were seasonal changes in: (1) the relation between maximum food intake and body weight; (2) the relation between water and food intake; (3) water, electrolyte, creatinine and urea balances.

MATERIALS AND METHODS

Animals

Animals were collected with live traps in the area of Red Feather Lakes, Colorado in 1980 (altitude 2438–2530 m). Animals were housed singly in cages (46 × 61 × 46 cm) in the animal care facilities of the Dalton Research Center. The initial series of studies were performed in 1981 with 18 animals approximately 2 years of age. The final series was performed in 1982 with six of the animals previously studied and were approximately 3 years of age.

When the animals were obtained they were dipped to eliminate ectoparasites (Kem–Dip, Vet Kem Co., Dallas, Texas). All animals were placed in quarantine for one month and provided with a 20% sulfaquinoxaline (Merck Chemical Division) in the drinking water to prevent or treat coccidiosis. Following the quarantine period they were placed in the regular animal house facility with a photoperiod of 12L:12D. At this time we attempted to feed them a commercial rabbit diet (Lab Rabbit Chow, Ralston Purina Co.) which was recommended for *Marmota monax* by Young and Sims (1979) and Lawless *et al.* (1982). We found that this species would not consume the diet and began to lose weight. We then changed to a commercial rat diet (Rodent Laboratory Chow, Ralston Purina Co.) which had been used in our earlier studies and found that unlike *monax*, *flaviventris* gained well and did not become excessively fat.

In addition to the rat chow the animals were provided with carrots once a week and vitamins (Vi-Sorbin, Norden Laboratories) in the drinking water once each week.

Following the first study and prior to the second one the animals were placed in a hibernaculum (6°C) and permitted to hibernate until early March. Cotton wool bedding was provided as nesting material. Food and water were provided *ad libitum*.

Body weight and food and water consumption

18 animals were used in this study (9 males and 9 females). Each animal was provided with 200 g of commercial rat diet blocks and 450 ml of water daily. The amount of food and water consumed was determined daily between 09:00 and 14:00 hr. Once each week (Monday) water bottles were replaced and 1 ml of a vitamin supplement, Vi—Sorbin, was added to each water bottle. Once each week (Thursday) 100 g of carrot were provided to each animal. The amount consumed was recorded on the following day. Animals were weighed every 2 weeks. The initial weight range of these animals was: males 2.52–6.95 kg, females 2.60–5.10 kg.

Body weight, food and water consumption and urine production

Three animals of each sex were used in this study. The animals were one year older and were randomly selected

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from the group that was used in the previous study. Only six animals comprised this study due to the large number of analyses necessary and because of the limited number of metabolic cages available. Food, water, carrots and vitamins were provided as indicated above. During the first week only five animals were studied. A sixth animal (male) was added in the second week.

Animals were housed individually in metabolism cages that were built for this study by the Dalton Research Center shop. Below the screen floor was a fine screen (fiberglass) to catch feces and food crumbs. A funnel-shaped box with a small opening at the bottom was formed of sheet stainless steel. This funnel was situated below the fine screen. Urine collection was made by placing a container below the funnel. The urine collecting containers had in them a layer of water-equilibrated mineral oil to prevent the evaporation of the urine sample (Zatzman and South, 1975). Urine volume was measured daily and an aliquot was frozen (−20°C) for later chemical analysis.

Animals were weighed every 2 weeks. The initial weight range was: males 3.8–5.23 kg, females 3.24–4.95 kg. At one month intervals a 4 ml blood sample was obtained by cardiac puncture from each of the sedated animals (ketamine 10 mg/kg i.m.). Blood samples were transferred to 5 ml tubes containing ammonium heparin. After centrifugation the plasma was frozen and stored for later analysis.

Chemical analysis

Chemical analysis was performed in the second study; only food and water consumption were determined in the first study.

At two week intervals the urine samples were thawed, mixed and the following analyses were performed: osmotic pressure by freezing point depression with a Fiske osmometer, urea nitrogen by the method of Marsh *et al.* (1965) and creatinine by an automated procedure developed by the Technicon Instruments Corp., and sodium and potassium by flame photometry. At the end of the study plasma samples were analyzed by the same methods. In addition, an aliquot of tap water, commercial rat diet and carrot were analyzed for sodium and potassium content. Sodium and potassium intake from carrots were included with the intake from the food eaten.

Analysis of the data

Daily consumption and excretion were corrected for body weight by assuming that the change in weight (measured bimonthly) was linear. A weekly average was computed for each animal.

Clearances (creatinine, urea, osmotic and free water) were calculated from the average daily excretion rate for the month divided by the average plasma concentration for that period. Weekly averages of daily consumption or excretion were analyzed statistically by the method of repeated measurements described by Gill and Hafs (1971). The method, which is an analysis of variance procedure, permits statistical evaluation of the effect of time as well as the effect of sex. The same statistical procedure was used for bimonthly body weights, monthly plasma concentrations and monthly clearance. Mean separation due to sex was ascertained using Fisher's least significant difference (LSD). Where appropriate, linear regression lines were calculated and the correlation coefficient was determined. Differences for each animal were calculated for water, Na and K between intake and excretion rates. These differences were also analyzed by the repeated measurements design.

RESULTS

Body weight, food and water consumption

Average body weights of males (Table 1 and Fig. 1) exceeded that of females ($P = 0.02$) throughout the period when measurements were made (8 March–23 August). Peak weight of males and females was achieved between late June and the third week in July (Table 1 and Fig. 1). There was no significant difference of food consumption between males and females when food consumption was corrected to body weight (Fig. 2, top panel). However, peak food consumption occurred in both sexes much earlier (29 March–24 May) than the maximum increase in weight. Water consumption, on a body weight basis, demonstrated the same pattern as did food consumption (Fig. 2, middle panel).

Table 1. Body weight, average food and water consumption by 18 marmots

		Body weight (kg)				Av. food consumption (g/day)				Av. water consumption (ml/day)			
		Males		Females		Males		Females		Males		Females	
Week	Date	Wt	SEM	Wt	SEM		SEM		SEM		SEM		SEM
1	3/5–3/11	4.36	0.56	3.72	0.27	62.2	9.3	77.9	12.8	128	25	128	18
2	3/12–3/18	4.37	0.52	3.81	0.24	63.4	8.6	79.9	14.3	138	41	134	22
3	3/19–3/25					66.4	7.8	81.9	12.9	127	21	129	21
4	3/26–4/1	4.37	0.51	3.86	0.24	91.6	9.9	101.2	14.3	193	47	182	21
5	4/2–4/8					96.3	9.0	113.6	18.2	152	28	191	20
6	4/9–4/15	4.62	0.53	4.12	0.25	108.6	9.9	110.2	12.2	191	26	213	23
7	4/16–4/22					104.1	11.3	106.7	12.1	192	26	206	24
8	4/23–4/29	4.76	0.54	4.29	0.26	117.8	12.3	116.8	13.0	211	40	205	25
9	4/30–5/6					119.4	11.8	99.3	3.9	216	34	168	14
10	5/7–5/13	4.95	0.55	4.39	0.33	122.0	14.7	99.2	6.4	231	34	186	26
11	5/14–5/20					126.6	15.1	98.2	11.3	228	37	163	20
12	5/21–5/27	5.19	0.59	4.61	0.27	120.6	18.1	101.0	11.5	228	43	150	25
13	5/28–6/3					114.2	19.6	97.2	15.5	212	44	139	26
14	6/4–6/10	5.39	0.61	4.61	0.27	104.9	16.8	81.0	17.0	187	41	124	28
15	6/11–6/27					97.1	16.8	68.2	15.0	145	35	93	22
16	6/18–6/24	5.45	0.64	4.62	0.31	92.8	17.7	58.2	15.6	145	41	69	17
17	6/25–7/1					87.2	18.2	46.8	14.4	124	37	63	18
18	7/2–7/8	5.50	0.67	4.62	0.33	81.6	19.8	44.8	12.9	117	40	44	13
19	7/9–7/15					66.9	17.5	28.8	9.2	107	36	28	10
20	7/16–7/22	5.46	0.68	4.54	0.34	57.0	13.9	27.0	9.6	89	35	21	8
21	7/23–7/29					40.3	10.2	20.1	6.4	63	27	11	4
22	7/30–8/5	5.29	0.66	4.37	0.36	39.5	11.5	16.4	6.0	59	29	6	2
23	8/6–8/12					32.8	9.0	9.0	2.7	50	20	4	1
24	8/13–8/19	5.33	0.65	4.30	0.33	32.9	9.0	6.2	2.4	41	22	5	2
25	8/20–8/26	5.32	0.63	4.33	0.35	19.8	5.7	5.6	1.6	34	17	4	3

*i.e. 5 March–11 March.

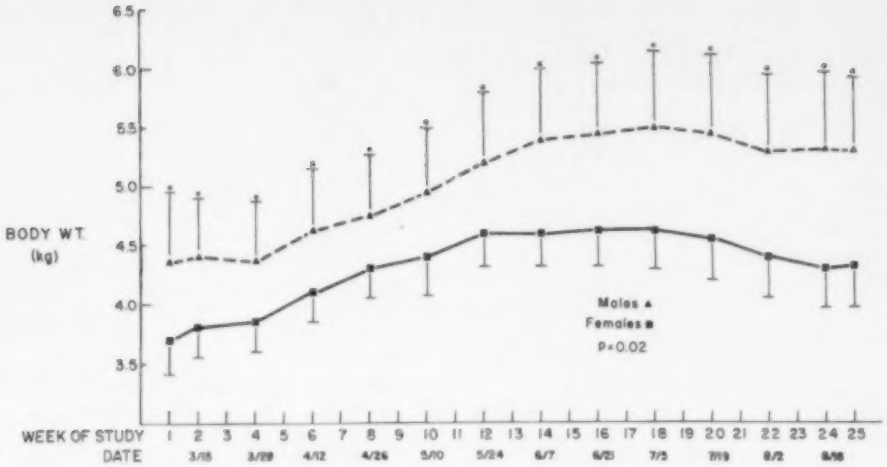

Fig. 1. Bimonthly body weights of *M. flaviventris*. ▲---▲ males ($N = 9$); ■——■ females ($N = 9$). *Difference between male and female weight are significant ($P < 0.02$). Verticasl bars are SEM.

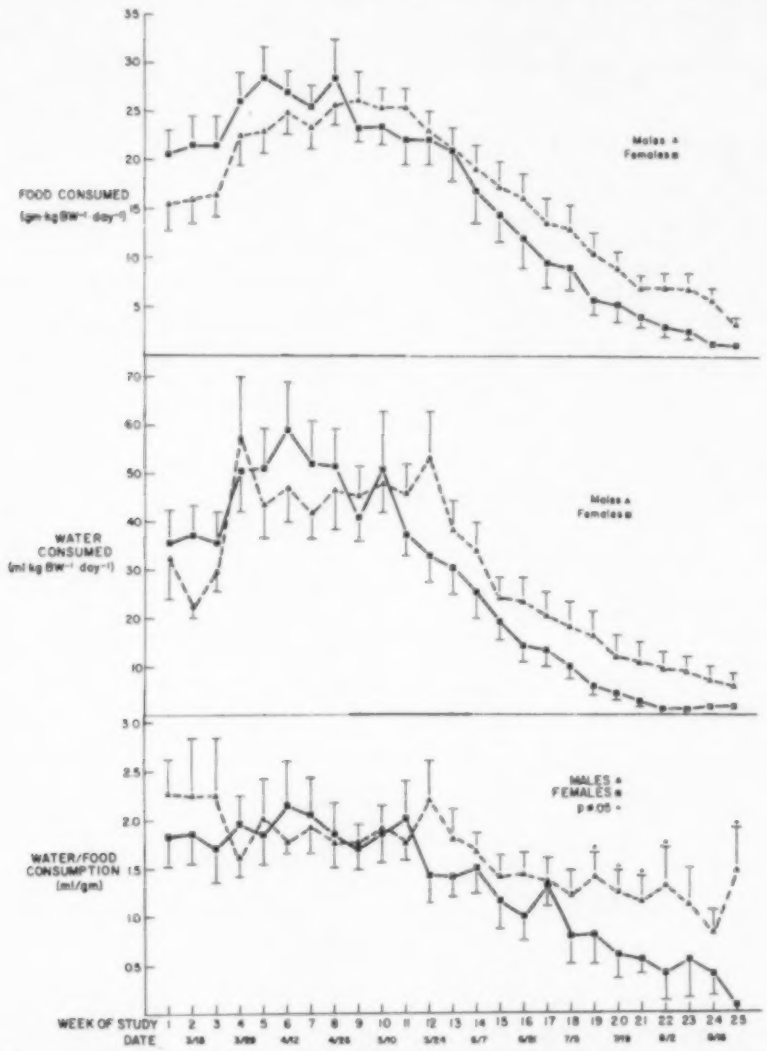

Fig. 2. Food and water consumption of 9 male and 9 female *M. flaviventris*. Upper panel—food consumption; middle panel—water consumption; lower panel—ratio of water consumption to food consumption. *Significant difference between males and females ($P < 0.05$). Vertical bars are SEM.

However, an analysis of the ratio of water:food consumption (Fig. 2, bottom panel) indicates that during late winter and early spring about 2 ml of water was consumed for each gram of food eaten; by late summer the ratio fell to 0.5–1.0 indicating that water consumption decreased faster than food consumption (see Fig. 2, upper and middle panels). In addition, this group of animals demonstrated that the ratio of water to food consumption decreased more in females toward late summer than males ($P < 0.05$). Food consumption, water consumption, body weight and ratio of water to food consumption changed significantly ($P < 0.001$) with time. By the end of this study the animals were lethargic and ready for hibernation.

Body weights, food and water consumption and urine production

This study was initiated about one month later than the first one because of delay in obtaining metabolism cages. One animal (male) was added to the study in the second week.

The average results are indicated in Table 2 and Figs 3–5. Since no differences were noted between the sexes the values in the table and figures are the overall averages.

The patterns of body weight, food and water consumption (Figs 3 and 5) of these older animals were similar to that observed in the initial study, although peak body weight occurred about 1 month earlier and averaged approximately 0.5 kg as compared to 1.0 kg in the younger marmots. Because of the small number of animals in this study there was no significant difference in the body weights of males as compared to females although the mean body weights of males exceeded that of females.

The ratio of water:food consumption demonstrated a pattern similar to that observed with the larger group of animals (compare Fig. 2, bottom panel and Fig. 3, middle panel). Except where noted, all measured values demonstrated significant changes with time.

Plasma constituents

Figure 4 contains the plasma concentrations of urea nitrogen (U), creatinine (Cr), sodium (Na), potassium (K) and the plasma osmotic pressure (P_{op}). All except Na ($P = 0.09$) and K ($P = 0.12$) concentrations demonstrated a change with time ($P < 0.01$) although the time of the minimum concentration of sodium and the potassium peak agrees with that found in a study with a larger animal population (Zatzman and South, 1981). Plasma urea concentrations gradually decreased from early April to the middle of August. On the other hand, both plasma creatinine concentration and osmolality of the plasma demonstrated an increase over the same period.

Intake and excretion of water and electrolytes

The intake of water and electrolytes and the excretion of these materials are indicated in Table 2 and Fig. 5. They all demonstrated a significant change with time ($P < 0.0001$).

As indicated in the inserts of Fig. 5, there was a reasonable balance between the consumption of water, Na and K and their excretion.

The same lot of commercial diet was used throughout the study and contained 0.127 mEq Na/g and 0.215 mEq K/g. Carrots contained 0.01 mEq Na/g and 0.07 mEq K/g. Tap water, which was provided for drinking, contained undetectable concentrations of Na and K.

The daily intake of water and the excretion of urine by each animal demonstrates an overall significant difference ($P < 0.0001$) between these two processes. Initially (11 April–21 April) there was a greater water intake than urine production (P values ranged from 0.0001 to 0.04). Late in the study (16 July–27 August) it appeared that urine loss exceeded water uptake; however, the differences were small (as were the absolute values), and were not significantly different from each other. Since there may have been a gradual dehydration of the animals due to a cumulative difference between water consumption and urine production, we tested (paired t-test) the difference between the sums of water consumed and urine produced during that period and found that the cumulative urine loss exceeded intake ($t = 2.88$; $P < 0.05$).

Potassium intake and excretion demonstrated the same pattern seen in water intake and urine excretion. Potassium intake exceeded excretion (P values between 0.02 and 0.0001) between weeks three and six (23 April–14 May). Although the slight excessive increase in K excretion was not significant, the total excretion from 11 May to 27 August exceeded the intake during the same period ($t = 2.78$; $P < 0.05$).

Although Na intake and excretion decreased from spring through late summer ($P < 0.0001$) there was no evidence of a significant difference between these processes ($P > 0.20$).

Osmotic, free water, creatinine and urea clearances

Osmotic and free water clearance, excretion and clearances of creatinine and urea are shown on Fig. 6. Osmotic clearance (upper panel) decreased dramatically throughout the study ($P < 0.0001$) and was accompanied by an increasing ($P < 0.001$) free water clearance. Free water clearance, however, never became positive, thus indicating a maintained but diminished water reabsorption. The maintenance of water reabsorption throughout the study is supported by the fact that urine osmolality never fell below 430 mOsm/kg (range 430–2500 mOsm/kg). The weekly average of urine osmolality remained surprisingly constant in the face of diminished urine flow (Table 2).

Creatinine excretion ($P < 0.0001$) and clearance ($P < 0.01$) decreased throughout the study (Fig. 6, middle panel). The general shape of the clearance pattern was similar to that found with exogenous creatinine clearance (Zatzman and South, 1981), but the absolute values were somewhat reduced. Urinary concentration of creatinine increased 4-fold by the end of the study (Table 2) while its excretion rate halved (Fig. 6).

The excretion of urea nitrogen (Fig. 6, lower panel) very closely paralleled food consumption ($r = 0.95$, $P < 0.001$) with a peak at week four (30 April), a

Table 2. Body weight, average food and water consumption and average urine production of 6 marmots

Week		Body wt (kg)	Food in.* (g/day)	Water in.* (ml/day)	$\dot{V}$† (ml/day)	U_{Na} (mEq/l)	U_K (mEq/l)	U_U (mg/ml)	U_{Cr} (mg/ml)	U_{OP} (mOsm/kg)	P_{Na} (mEq/l)	P_K (mEq/l)	P_U (mg/ml)	P_{Cr} (mg/ml)	P_{OP} (mOsm/kg)
1	4/9‡	4.51 ± 0.35	94 ± 11	166 ± 18	109.26 ± 6.35	120.2 ± 5.99	196.1 ± 12.00	13.63 ± 0.93	0.76 ± 0.07	1278.32 ± 55.18	148 ± 0.60	4.28 ± 0.07	0.28 ± 0.02	0.008 ± 0.001	308 ± 1.28
2	4/16	4.71 ± 0.40	109 ± 13	174 ± 20	106.55 ± 13.30	123.2 ± 10.71	200.3 ± 14.34	16.16 ± 0.81	0.79 ± 0.05	1362.74 ± 80.72					
3	4/23		118 ± 18	169 ± 27	105.52 ± 18.71	126.7 ± 12.89	196.8 ± 20.24	17.83 ± 1.53	0.75 ± 0.09	1424.36 ± 126.59					
4	4/30	4.95 ± 0.42	124 ± 18	161 ± 27	110.07 ± 18.16	138.6 ± 9.13	217.9 ± 13.78	19.88 ± 1.22	0.76 ± 0.10	1575.67 ± 77.63					
5	5/7		95 ± 13	99 ± 21	76.07 ± 16.26	138.2 ± 9.15	214.4 ± 9.95	25.65 ± 1.02	1.19 ± 0.16	1637.85 ± 75.69	149 ± 1.17	4.95 ± 0.35	0.34 ± 0.02	0.008 ± 0.0006	317 ± 1.31
6	5/14	5.04 ± 0.43	89 ± 18	126 ± 30	90.38 ± 26.27	145.2 ± 19.81	220.4 ± 24.13	21.35 ± 1.74	1.11 ± 0.20	1579.49 ± 127.92					
7	5/21		79 ± 15	105 ± 29	84.08 ± 23.30	135.9 ± 11.24	241.3 ± 23.89	20.72 ± 1.94	1.14 ± 0.20	1585.24 ± 135.16					
8	5/28	5.09 ± 0.47	74 ± 18	75 ± 21	66.09 ± 17.55	140.5 ± 8.61	256.9 ± 17.01	23.07 ± 1.44	1.34 ± 0.20	1781.88 ± 94.58					
9	6/4		45 ± 16	46 ± 22	45.46 ± 17.96	158.78 ± 12.95	259.6 ± 24.06	26.12 ± 1.44	1.97 ± 0.30	1814.69 ± 98.68	148 ± 1.30	6.5 ± 1.13	0.32 ± 0.02	0.009 ± 0.006	316 ± 1.82
10	6/11	5.00 ± 0.50	50 ± 15	59 ± 29	42.12 ± 13.62	178.2 ± 12.9	293.9 ± 27.16	23.03 ± 1.70	1.76 ± 0.33	1834.1 ± 126.94					
11	6/18		40 ± 14	48 ± 26	34.38 ± 11.58	198.08 ± 30.68	293.8 ± 30.49	24.68 ± 1.83	2.06 ± 0.40	1827.7 ± 129.87					
12	6/25	4.92 ± 0.52	37 ± 10	38 ± 11	32.30 ± 9.67	163.5 ± 24.26	297.8 ± 38.96	20.67 ± 1.79	1.95 ± 0.43	1751.15 ± 146.12					
13	7/2		32 ± 10	31 ± 9	33.50 ± 8.04	144.0 ± 14.16	251.4 ± 29.40	22.15 ± 2.77	2.48 ± 0.46	1612.16 ± 161.64	149 ± 0.70	5.02 ± 0.27	0.25 ± 0.03	0.009 ± 0.0004	317 ± 2.25
14	7/9	4.88 ± 0.52	25 ± 11	18 ± 14	23.48 ± 4.48	159.5 ± 26.71	262.5 ± 27.22	20.13 ± 2.43	2.65 ± 0.58	1607.14 ± 145.15					
15	7/16		19 ± 6	15 ± 5	19.36 ± 4.10	130.68 ± 18.28	270.4 ± 34.85	18.23 ± 2.09	2.52 ± 0.29	1639.95 ± 151.12					
16	7/23	4.81 ± 0.51	13 ± 3	7 ± 2	15.42 ± 3.68	105.7 ± 16.79	218.3 ± 16.68	19.40 ± 2.44	3.64 ± 0.55	1508.55 ± 118.13					
17	7/30		12 ± 3	11 ± 2	10.68 ± 2.00	110.9 ± 9.32	253.3 ± 25.68	21.93 ± 2.54	4.31 ± 0.63	1710.92 ± 154.95	152 ± 1.18	4.78 ± 0.18	0.19 ± 0.01	0.011 ± 0.0008	314 ± 1.49
18	8/6	4.80 ± 0.45	15 ± 3	5 ± 1	16.00 ± 2.01	137.2 ± 24.56	248.0 ± 27.50	16.23 ± 2.87	2.98 ± 0.29	1386.26 ± 168.09					
19	8/13		11 ± 2	6 ± 2	12.62 ± 1.59	91.0 ± 11.58	229.4 ± 13.66	16.85 ± 2.41	3.38 ± 0.37	1410.31 ± 124.45					
20	8/20	4.64 ± 0.52	10 ± 2	7 ± 2	11.54 ± 1.51	121.6 ± 13.02	212.7 ± 22.34	15.40 ± 2.03	3.47 ± 0.32	1411.68 ± 162.11					
21	8/27	4.70 ± 0.44	14 ± 5	10 ± 5	15.15 ± 1.94	124.2 ± 15.46	267.2 ± 30.07	18.05 ± 1.66	3.19 ± 0.35	1449.96 ± 117.14					
	9/3										148 ± 1.05	4.31 ± 0.13	0.23 ± 0.02	0.012 ± 0.001	317 ± 1.36

*Food or water intake.
†Urine flow.
‡Date (month/day).

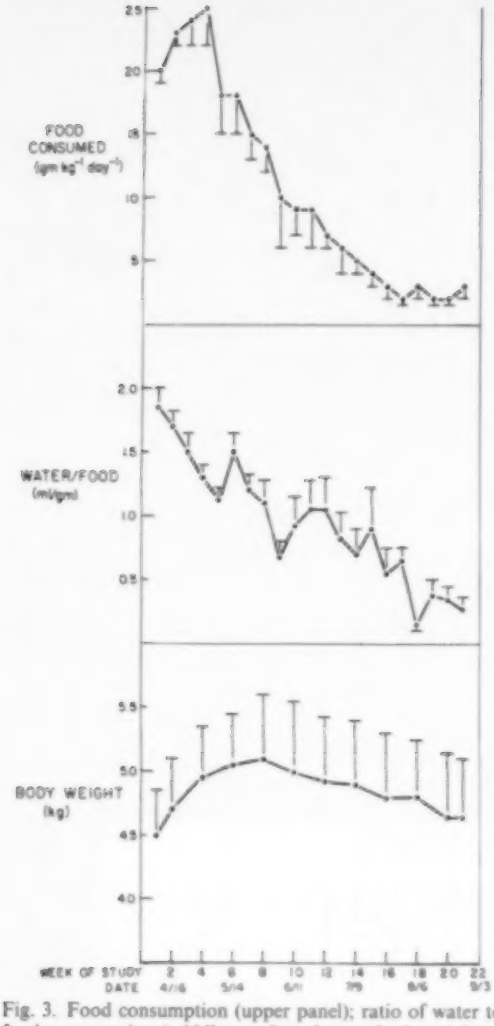

Fig. 3. Food consumption (upper panel); ratio of water to food consumption (middle panel) and mean body weight of 6 *M. flaviventris* (3 males, 3 females). Since no sex differences were noted, figures contain overall means. Vertical bars are SEM.

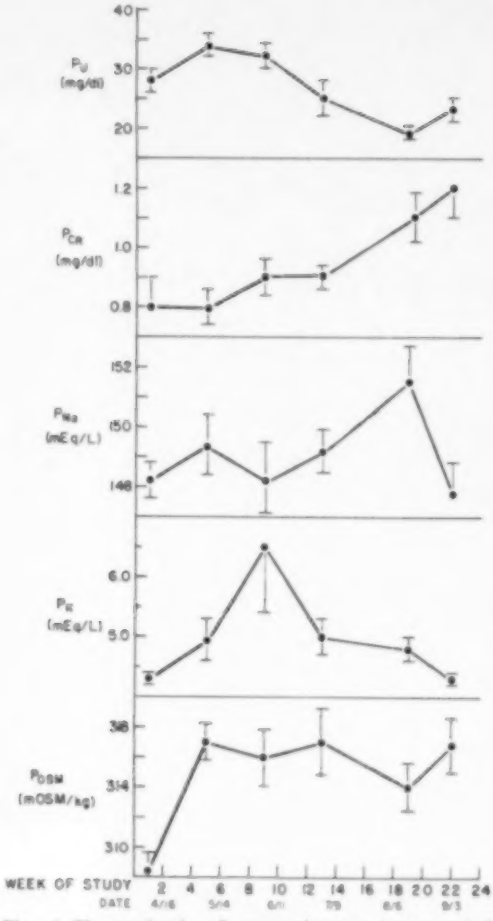

Fig. 4. Plasma levels of: urea nitrogen (upper panel), creatinine (Panel 2), sodium (Panel 3), potassium (Panel 4) and osmolality (bottom panel). Vertical bars are SEM.

decrease to week sixteen (23 July) and then a plateau for the remaining time of the study.

Urea clearance, however continued to diminish throughout the study (Fig. 6, lower panel), due principally to the reduction in urine flow since urine urea nitrogen concentrations remained relatively stable (Table 2).

DISCUSSION

Seasonal alteration of body weights of hibernants (Pengelly and Fischer, 1963: Davis, 1976; Fall, 1971; Armitage *et al.*, 1976; Young and Sims, 1979; Hock, 1969) including *M. monax* (Davis, 1976; Fall, 1971; Armitage *et al.*, 1976; Snyder *et al.*, 1961) and *M. flaviventris* (Armitage *et al.*, 1976; Hock, 1969) have been documented. In general, male marmots are larger than females (Armitage *et al.*, 1976) in agreement with our results (Fig. 1). There are, however, discrepancies in the literature concerning the time at which peak weight is achieved. Studies in the field (Davis, 1976; Armitage *et al.*, 1976; Snyder *et al.*, 1961) indicate that both *M. monax* and *M. flaviventris* continue to increase body weight until hibernation ensues. Captive animals, however, demonstrate peak weight sometime before hibernation begins and actually lose weight prior to the hibernation period (Davis, 1976; Young and Sims, 1979). This pattern was reproduced by our studies (Fig. 1 and Fig. 3). The larger group of animals (2 years old) reached peak weight late in June or early in July, while the older animals reached peak weight about a month earlier. Thus, under conditions of constant photoperiod our animals demonstrated an 11 month cycle of weight gain as investigators have found with other hibernants (Pengelley and Fisher, 1963; Davis, 1976).

The literature contains no data on food and water consumption of *M. flaviventris*. There is some variability in the literature concerning feeding patterns of woodchucks. One study indicates a gradual increase in food intake until May to July and then a gradual decrease in food consumption until hibernation starts (Fall, 1971). In another by Davis (1976) food consumption reached its maximum level in August under conditions of decreasing day length. A group of

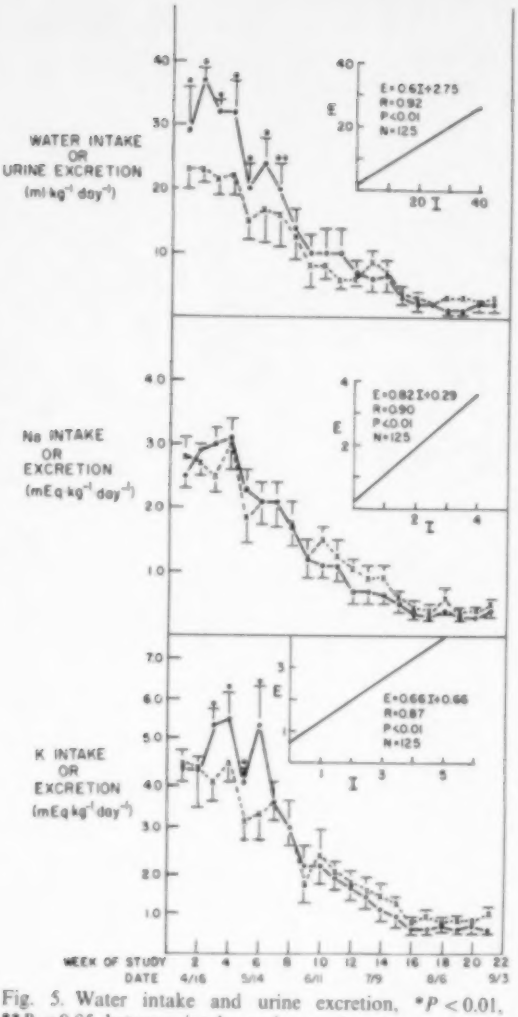

Fig. 5. Water intake and urine excretion, $*P < 0.01$, $**P < 0.05$ between intake and excretion (upper panel); sodium intake and excretion (middle panel); potassium intake and excretion, $*P < 0.02$, between intake and excretion (lower panel); ●——● intake, × --- × excretion. Vertical bars are SEM.

animals under constant conditions of L:D of 16:8 continued to increase food consumption until mid September. In a recent study, in which light approximated seasonal cycles, Young and Sims (1979) found that maximal food consumption occurred in mid May followed by a gradual decline to September when food consumption ceased. This latter pattern in the woodchuck is supported by studies that indicate maximal metabolic rates during May (Bailey, 1965). Maximal food consumption of *M. flaviventris* occurs earlier than in *monax* (Figs 2 and 3) and may have been influenced by the shorter natural feeding season of these animals (Armitage *et al.*, 1976). An unusual finding in our study and that of Young and Sims (1979) is the continued increase of body weight for a month or more beyond the peak food consumption.

We found, as did others working with *M. monax*, that food consumption of males was slightly higher but not significantly different from that of females either on the basis of intake per day or intake per kg of body weight per day (Figs 2 and 3).

No studies have been reported of water consumption in hibernants under laboratory conditions. Water consumption by *M. flaviventris* generally follows food consumption (Figs 2 and 5), but decreases more than food consumption between April and August. In the study with the larger group of animals the water:food consumption ratio decreased more in the female than male population. Decreased water consumption was associated with a decrease in urine flow and osmotic clearance (Fig. 6).

Although free water clearance remained negative, water reabsorption diminished by a factor of seven and may have contributed to the gradual rise in plasma osmotic pressure since respiratory (Benedict and Lee, 1938) and fecal water loss may have been significant. In addition to the relative decrease in total body water, the plasma osmotic pressure could have been increased due to an increase in plasma albumin which was demonstrated in the 13-lined ground squirrel throughout the late spring and into the winter months (Galster and Morrison, 1966). It appears that one of the preparative features for hibernation is a relative dehydration of the animal which is supported by the work of Jameson and Mead (1964) who found a 13% decrease in the total body water of *C. lateralis* prior to hibernation. Excess intake of water in the initial part of the study may be necessary for rehydration of the marmot following hibernation. In addition, since some growth occurs after emergence in the spring, the positive K balance may be associated with the increased requirement for incorporation into new cellular elements.

There were significant alterations in the relation between K intake and excretion. Potassium loading was apparent at the time of rehydration and a cumulative loss of K prior to hibernation. Due to the relative stability of plasma K (P_K) in the face of larger alterations of intake or excretion, P_K did not show significant changes. Sodium balance was better maintained than that of either water or potassium, with no apparent excesses in either intake or excretion. The obvious reciprocal relation between P_{Na} and P_K demonstrated in our previous study (Zatzman and South, 1981) is not as clear. However, the minimum P_{Na} coincides with the peak of P_K at about the same time that these changes occurred in the report mentioned. We hoped to clarify the process which produced those changes in plasma electrolytes. They cannot be accounted for by alterations in the relation between intake and output. In addition, urine concentrations of Na and K (Table 2) do not help in the determination of the factors responsible for the alteration of plasma K and Na noted previously. Urinary Na and K concentrations tended to be lowest at the initiation of the study when urine flow was greatest and at the end when urine flow was lowest. Unfortunately, no data are as yet available of seasonal plasma aldosterone concentrations which may play an important role in governing the P_{Na} and P_K levels.

The decrease noted in plasma urea nitrogen levels are essentially continuous with those we observed during hibernation of this species (Kaster *et al.*, 1978). A high correlation between food intake and the excretion of urea nitrogen ($r = 0.95$) argues

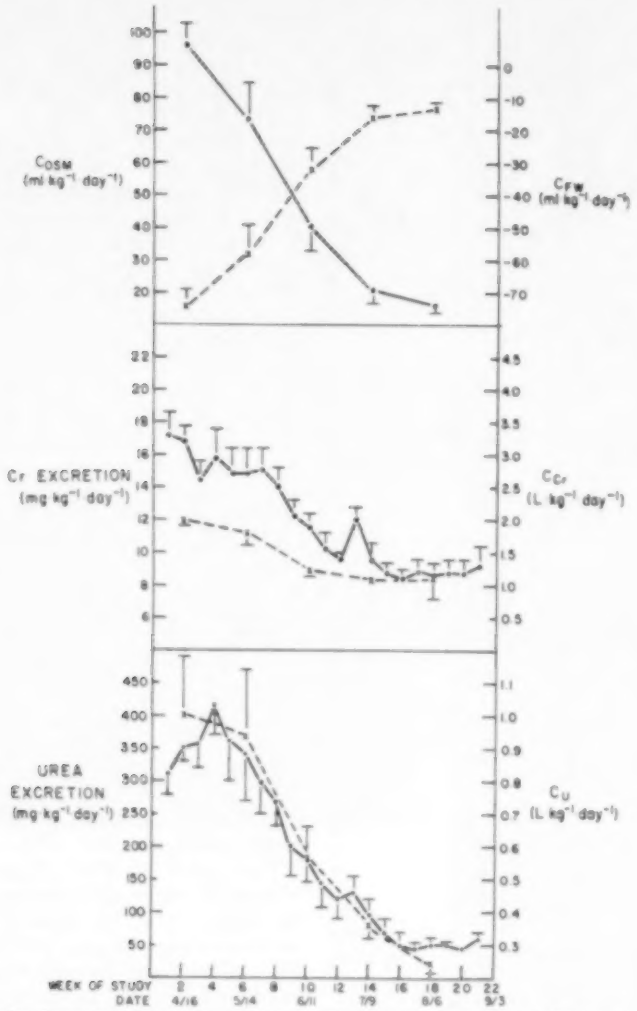
Cosm (ml kg⁻¹ day⁻¹)
CFW (ml kg⁻¹ day⁻¹)
Cr EXCRETION (mg kg⁻¹ day⁻¹)
CCr (L kg⁻¹ day⁻¹)
UREA EXCRETION (mg kg⁻¹ day⁻¹)
CU (L kg⁻¹ day⁻¹)
WEEK OF STUDY 2 4 6 8 10 12 14 16 18 20 22
DATE 4/16 5/14 6/11 7/9 8/6 9/3

Fig. 6. Osmotic clearance, ●——●; and free water clearance, × --- × (upper panel); creatinine excretion, ●——●; and creatinine clearance, × --- × (middle panel); urea excretion, ●——●; and urea clearance, × --- × (lower panel). Vertical bars are SEM.

against urea recycling which has been implicated in rodent hibernant metabolic activity (Riedesel and Steffen, 1980). Rather, urea clearance and excretion rate appear to be much more influenced by food intake than alteration in renal function. This is similar to the effect seen in humans with varying degrees of renal insufficiency on differing protein diets (Dossetor, 1966; Kopple and Coburn, 1974). Plasma creatinine concentration, on the other hand, is sensitive to alterations in renal function during the time that muscle mass is relatively constant (Dossetor, 1966). Since glomerular filtration rate diminishes throughout the entire study period (Zatzman and South, 1981 and see Fig. 6, middle panel) increasing plasma creatinine levels are not surprising.

In summary, we have examined the food and water consumption, urine production, urine and plasma constituents of some electrolytes, creatinine and urea. Shortly after arousing from hibernation water and food consumption increase with a greater intake of water than food. During this period water and potassium intake exceeded urinary excretion. Plasma osmotic pressure was low, as was plasma creatinine. Urea, creatinine and osmotic clearances were high; free water clearance was very low. During this period the marmot appeared to be rehydrating, protein intake and thus urea nitrogen excretion and plasma urea nitrogen were high and glomerular filtration rate was at its maximum (Zatzman and South, 1981).

As the season progressed from late winter to late summer, food and water intake diminished. Water intake fell faster than food consumption, free water clearance became less negative and urine production exceeded water intake; total body water probably decreased (Jameson and Mead, 1964). Associated with the decreased food consumption was a fall in urea nitrogen excretion, clearance and plasma level. Glomerular filtration rate reached its nadir by the end of the study in September with a concomitant decrease in creatinine excretion and clearance and an increase in plasma creatinine concentration and plasma osmotic pressure.

Alteration of plasma electrolytes cannot be as clearly delineated because of unknown hormonal changes that may occur during this cycle of events.

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STRESS- AND (1–24)ACTH-INDUCED ALTERATIONS IN ADRENAL AND TESTICULAR STEROIDOGENESIS IN MONGOLIAN GERBILS (*MERIONES UNGUICULATUS*): COMPARISON OF PLASMA LEVELS, TISSUE CONTENT AND *IN VITRO* SECRETION

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Abstract—1. Plasma glucocorticosteroid levels were significantly elevated 1 hr after confinement stress or (1-24)ACTH administration.

2. Both adrenal content and *in vitro* secretion of glucocorticosteroids and progesterone from adrenals of stressed or (1-24)ACTH-injected animals were higher than values measured in controls.

3. Neither adrenal testosterone content nor output of testosterone or progesterone from superfused testes were changed.

4. Significant correlations were obtained between glucocorticosteroid plasma levels and corresponding adrenal content/*in vitro* secretion, adrenal progesterone content and output, and between adrenal glucocorticosteroid and progesterone content.

INTRODUCTION

The production of steroids by *in vitro* superfusion of endocrine organs follows a reproducible pattern which is characteristic of the species and the physiological status of the animal at the time the gland is removed (adrenal: for ref. see Fenske, 1983; pituitary: for ref. see Stern and Conn, 1981; Whitehead *et al.*, 1982). Little comparable research has been carried out to date on the Mongolian gerbil, a species whose adrenal and testicular physiology differs in several aspects from that of other laboratory animals (Nickerson, 1971, 1972; Nickerson and Curtis, 1969; Probst and Fenske, 1982; Fenske and Probst, 1982; Fenske, 1983).

The present work was undertaken to determine adrenal and testicular activity by measuring *in vivo* (plasma concentrations) and *in vitro* (tissue content, secretion) parameters in normal, stressed (1-hr confinement) or (1-24)ACTH-treated animals.

MATERIALS AND METHODS

Substances

HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid), TC medium 199 + Earle's salts, fetal calf serum and non-radioactive steroids were purchased by Serva (Heidelberg, FRC), 1-methyl-3-isobutyl-xanthine (MIX) from Sigma, ^{3}H(1,2,6,7)*N*-steroids from NEN-Chemicals (Dreieichhain, FRG).

Animals

Mongolian gerbils (*Meriones unguiculatus*, 4 months of age) were used. They were maintained singly in cages under controlled temperature (22 ± 1°C) and lighting (light from 7.30 to 19.30) conditions. Food (gerbil pellet; Altromin, Braunschweig, FRG) and tap water were available *ad libitum*. Groups of 5–7 animals were randomly assigned to each of the following conditions: (1) Animals were placed in confinement chambers, (2) and (3) animals were exposed to ether vapor for 60 sec and were injected intraperitoneally with saline (0.2 ml) or 6 IU (1-24)ACTH (Acortan simplex, Ferring, Kiel, FRG). Sixty minutes after the beginning of the respective treatment, the animals were decapitated. Control animals were sacrificed within 1 min after removal from the animal room. Blood was collected in heparinized tubes, centrifuged, and the plasma kept at −25°C until assayed. Corresponding adrenal/testicular tissue was either frozen quickly to −75°C or was superfused over 60 min. For superfusion experiments, right adrenals or testes were dissected free of fat, were quartered (adrenals) or decapsulated and halved (testes), transferred into incubation flasks (retention volume: 0.2 ml (adrenals) or 1.4 ml, testes) and were superfused with medium (TC medium 199 containing 1% fetal calf serum, 25 mmol HEPES, 0.01 mmol MIX) at a rate of 0.4 ml/min. Additional details of the superfusion system were described elsewhere (Fenske, 1983).

Radioimmunoassays

Steroid levels in plasma or tissue were determined after extraction; amounts released *in vitro* were measured in unextracted aliquots of supernatant. The antisera, raised in rabbits, were generously supplied by Dr. F. Bidlingmaier (Division of Pediatric Endocrinology, Children's Hospital of the University of Munich, FRG). The detailed procedures of assay performance and their validation has been reported earlier (Sippell *et al.*, 1978; Fenske and Probst, 1982).

Results are presented as mean values ± SEM. Statistical analyses were performed using the Mann–Whitney *U*-test.

RESULTS

Control animals had plasma levels of 296 ± 63 ng/ml (glucocorticosteroids, $N = 7$), 2.43 ± 1.44 ng/ml (progesterone) and 1.43 ± 0.26 ng/ml (testosterone). Confinement or (1-24)ACTH administration resulted in significantly elevated glucocorticosteroid plasma levels (Fig. 1). In contrast, neither testosterone nor progesterone plasma levels were significantly changed.

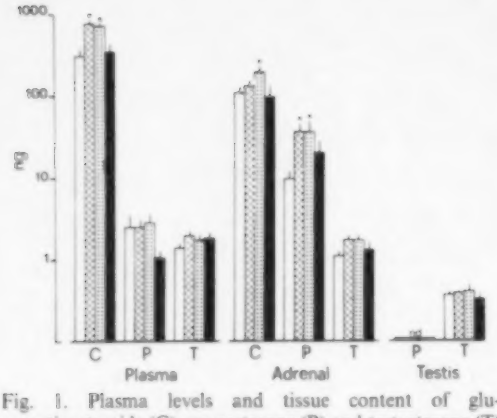

Fig. 1. Plasma levels and tissue content of glucocorticosteroids (C), progesterone (P) and testosterone (T) in gerbils. □ Control animals ($N = 7$). ▩ Animals confined for 60 min ($N = 6$). ▦ Animals injected with 6 IU (1–24)ACTH under ether anaesthesia and killed 60 min later ($N = 6$). ■ Animals injected with saline under ether anaesthesia and killed 60 min later ($N = 5$). Steroid amounts are expressed in ng/ml (plasma) or ng/10 mg tissue (adrenal, testis); nd, not detectable. Mean ± SEM, $\times \alpha < 0.01$, U-test.

Compared to control animals, an increase of adrenal glucocorticosteroid and progesterone content was found in stressed or (1-24)ACTH-treated animals (Fig. 1). Similarly, output of glucocorticosteroids and progesterone from superfused adrenals of confined or (1-24)ACTH-injected animals was significantly increased (Fig. 2). In addition, glucocorticosteroid plasma levels and corresponding adrenal content ($r = 0.59$, $P < 0.01$)/output ($r = 0.52$, $P < 0.01$), adrenal progesterone content and output ($r = 0.63$, $P < 0.001$) and adrenal glucocorticosteroid/progesterone content ($r = 0.76$, $P < 0.001$) were significantly correlated.

In contrast to changes of adrenal activity, no significant differences in either content (Fig. 1) or output (Fig. 3) of testosterone or progesterone from testes of animals of the four experimental groups existed.

DISCUSSION

In subhuman primates, as well as in small laboratory animals restrainment/confinement represents a very strong stress factor which has a pronounced effect on plasma steroid levels. Such changes occur in the levels of glucocorticosteroids which increase rapidly due to the applied stress factor. The present study confirms this type of response in gerbils and shows that also glucocorticosteroid and progesterone content/output of adrenals follows the same pattern. The concentrations of progesterone, which is a cortisol/corticosterone precursor, seem to follow closely the levels of glucocorticosteroid levels whatever changes those undergo (rat: Holzbauer and Newport, 1967; Fajer *et al.*, 1971; Varga and Greenwald, 1979; pig: Blatchford *et al.*, 1978; Fenske *et al.*, 1981; monkey: Goncharov *et al.*, 1979; gerbil: this study, Figs 1 and 2).

In contrast to changes of adrenal gland activity, the activity of the testes, as argued from plasma, tissue and *in vitro* secreted amounts of testosterone and progesterone, is not changed by exposure to stress

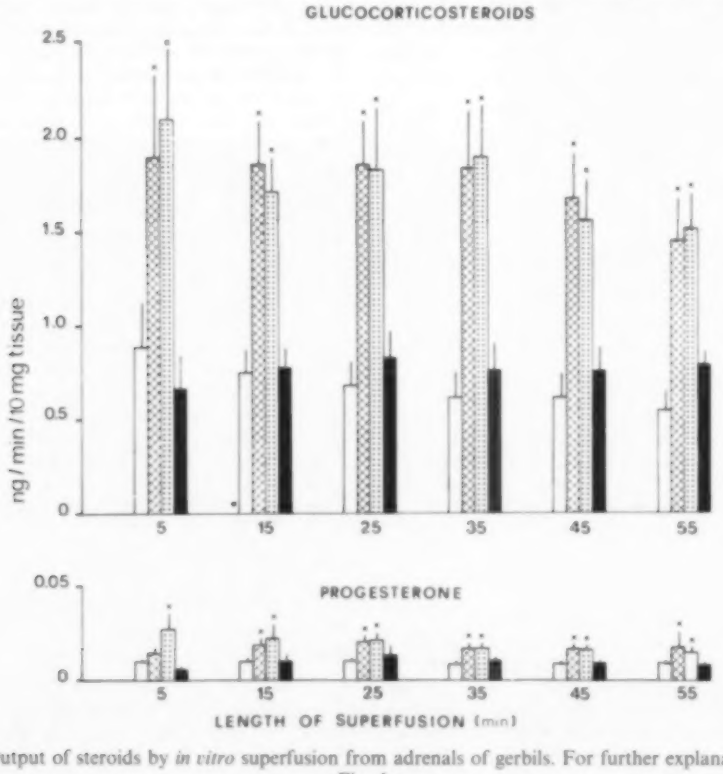

Fig. 2. Output of steroids by *in vitro* superfusion from adrenals of gerbils. For further explanations, see Fig. 1.

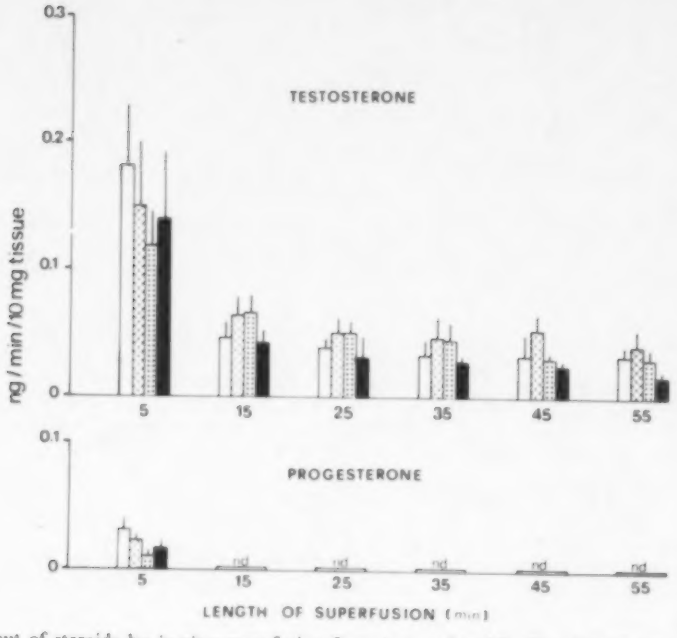

Fig. 3. Output of steroids by *in vitro* superfusion from testes of gerbils. For further explanations, see Fig. 1.

factors such as handling (Fenske, 1983), exposure to a novel environment (Fenske, 1983), confinement or (1-24)ACTH administration (this study). These results contrast to earlier work in other species which reported a marked fall of plasma levels or testicular testosterone content (Fariss *et al.*, 1969; Matsumoto *et al.*, 1970; Bliss *et al.*, 1972; Cartensen *et al.*, 1973; Saez *et al.*, 1977; Collu *et al.*, 1979; Welsh and Johnson, 1981) following exposure to various stress stimuli. Goncharov *et al.* (1979) concluded from their work on baboons that testosterone decline under stress conditions may have a common mechanism in mammalian species and that changes of testosterone represent a very distinct indicator of stress in males. Concerning the results in gerbils, it should be emphasized that the time interval between the beginning of exposure to stress factors/(1–24)ACTH administration may be too long/short to induce significant changes of testosterone and/or progesterone levels. Further studies are necessary to determine whether the secretory activity of the gerbil testes can be altered by prolonged exposure to stress factors or varying (1–24)ACTH doses.

Several points emerge from the present work that deserve particular emphasis. First, the *in vitro* approach reflects in part the *in vivo* activity of the adrenal gland, as is evident from the correlation between plasma and content/output of glucocorticosteroids and progesterone. Second, while measurement of tissue amounts of steroids is complicated by extraction steps and incomplete recovery (for ref. see Mathews *et al.*, 1981) and determination of changes of plasma steroid concentrations is difficult in unanaesthetized small laboratory animals, *in vitro* superfusion is a practical, efficient and relatively simple technique to measure change of adrenal activity. Amounts released during superfusion are high and secretory profiles can be easily determined by radioimmunoassay. By this technique, central nervous system and metabolic influences are excluded and dynamic changes after either *in vivo* or *in vitro* stimulation can be monitored over several hours.

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THE RELATIONSHIP BETWEEN MOLTING, REPRODUCTION, AND A HEMOLYMPH FEMALE-SPECIFIC PROTEIN IN THE LOBSTER, *HOMARUS AMERICANUS*

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Abstract—1. A female-specific protein (FSP), immunologically identical to oocyte lipovitellin, is present in the hemolymph of vitellogenic female lobsters (*Homarus americanus*).

2. FSP titers increase in the hemolymph during vitellogenesis, but the levels are maximum well prior to oviposition.

3. FSP does not appear in the hemolymph during the winter months, while the ovaries are quiescent, nor does it appear in females that are in late pre-molt stages, when vitellogenesis is suppressed prior to ecdysis.

4. It thus seems that the titer of hemolymph FSP is a good index of vitellogenesis, and that FSP is probably an extra-oocytic precursor of lipovitellin in transit to the oocyte.

INTRODUCTION

Both molting and reproduction in crustaceans occur on a seasonal timetable and are, moreover, integrated since most crustaceans continue to molt and grow after reaching sexual maturity. In female crustaceans, ovarian maturation and molting must occur at appropriate times, since the newly laid egg mass is carried on the maternal abdomen, and an inopportune molt would lead to a loss of these eggs. The North American lobster, *Homarus americanus*, is a valuable resource, and one might expect that a good deal of information might exist on both the molting and reproductive physiology. In reality, a good deal of the available information on molting and reproduction in *Homarus americanus* is based largely on field studies (Squires, 1970; Ennis, 1971; Krouse, 1973) which, in general, have largely been unable to define, in detail, the relationship between molting and oviposition, since these field studies were conducted by making single observations on a large number of animals. In this study, we have made sequential observations on individual lobsters maintained in the laboratory over two full seasons, and, thus, have been better able to define the timing of molting and oviposition in individual animals.

It is revealing to note that, although molting and reproduction in crustaceans are equally important and interesting events, reviews of crustacean physiology have historically not treated them as such (for example, see Carlisle and Knowles, 1959; Highnam and Hill, 1969; or Tombes, 1970). These reviews have extensive coverage of ecdysis and its control, but little as to the control or timing of reproductive events, although this imbalance has been redressed recently (Aiken and Waddy, 1976). It seems clear that this discrepancy has arisen since there exist several methods, in particular, pleopod sampling (Stevenson, 1972; Aiken, 1973), for "staging" incremental periods of proecdysis, whilst an equivalent method for monitoring reproductive events has not been practised to the same extent. Ovarian and testicular samples can be taken, but these invasive techniques do not leave the animal intact, and further, damage the very organ which is being studied.

In our studies on molting and reproduction in the female lobster, we were able to quantitate the levels of a female-specific protein (FSP) in the hemolymph, which is immunologically identical to lipovitellin, the major egg yolk protein as described by Wallace *et al.* (1967). This protein had been first reported to exist in *Homarus americanus* by Barlow and Ridgway (1969). We found that the levels of the FSP showed good correlation to the timing of vitellogenesis. This study describes, in detail, the occurrence and properties of the FSP in relation to molting and vitellogenesis in female lobsters. We feel that the FSP provides a rapid and reliable index of ovarian growth, and, coupled with the existing methods for monitoring proecdysis, will be a useful tool in experimental studies of lobster physiology.

MATERIALS AND METHODS

Experimental animals

Lobsters (*Homarus americanus*, Milne-Edwards) were obtained from retailers in New Brunswick and Nova Scotia, Canada. The lobsters were originally caught in the Northumberland Strait region, south-west of Prince Edward Island, Canada. Lobsters were kept in community tanks, and were fed a mulch of shrimp, herring, sea urchin and cod. Stages of proecdysis were determined by examination of fresh, severed pleopod tips according to the criteria established by Aiken (1973). Ovarian stages were determined by examining freshly dissected ovaries.

Hemolymph samples

Lobster hemolymph samples (up to 3 ml per sample) were obtained by syringe from just under the ventral abdominal membrane of the first or second abdominal segment. The fresh hemolymph sample was immediately placed into a centrifuge tube, and swirled gently until a visible clot formed. The tubes were left to stand undisturbed at 4°C overnight to ensure complete clotting. The clot was loosened with an applicator stick, and the clear serum was obtained by a 10–15 min centrifugation at 10,000 *g*. Serum was either used fresh for protein determinations and electrophoresis, or frozen at −20°C.

Isolation and purification of lipovitellin

Fragments of ovarian tissue at various stages of vitellogenesis were dispersed in a Potter–Elvejehm homogenizer in 10 volumes of ice-cold 0.5 M NaCl containing 5 mM EDTA* (sodium salt). The homogenates were centrifuged, in the cold, at 20,000 *g* for 20 min and the supernatants used for both electrophoresis or subsequent purification. The lipovitellin fraction was isolated from the 20,000 *g* supernatants using the ammonium sulphate precipitation technique described by Wallace *et al.* (1967) except that centrifugations were reduced to 20,000 *g* for 30 min at 0–5°C. The bulk of the lipovitellin precipitated at 67% saturation of ammonium sulphate at 0°C. Purity of the resulting preparations was checked electrophoretically.

Protein determinations

Total serum or ovarian protein levels were determined using the method of Lowry *et al.* (1951) or the Biuret method of Henry *et al.* (1957).

Electrophoresis

Lobster serum or ovarian proteins were separated electrophoretically on standard 7% acrylamide gels, poured into 90 × 5 mm glass tubes (Davis, 1964). All separations were carried out at 10°C with a reservoir buffer of 0.06 M Tris–glycine at pH 8.8. The volume of the sample was always less than 5 μl and was adjusted so that between 100 and 180 μg of protein were present.

Gels were stained by immersion in 0.5% Amido black in 7% aqueous acetic acid for 1–8 hr. Gels were destained electrophoretically and stored for subsequent quantification in 7% acetic acid. Some gels were stained with Sudan Black B (for lipid) or with the periodic acid–Schiff reagent (for carbohydrates) according to the methods described in Sargent (1969). Stained gels were scanned on a Photovolt Densicord densitometer that provided gel scans that were expanded 1:5 with respect to the original gel dimension. Areas under the curves were estimated by using the Integrator device attached to the densitometer. This device was shown to measure protein concentrations in a linear fashion as checked with a series of standard protein gels.

Immunological analysis

The precipitated lipovitellin fractions (in 67% ammonium sulfate) were resuspended in, and thoroughly dialyzed against 0.5 M NaCl at 5–10°C. Anti-lipovitellin antibodies were raised against this fraction by injecting rabbits with 1 ml each of a 5 mg/ml solution of protein and Freund's complete adjuvant. Three injections were administered over a three-week period. About ten days after the last injection, the rabbits were bled via a peripheral ear vein, and the presence of anti-lipovitellin confirmed using the ring precipitin test as described by Campbell *et al.* (1970). Samples of rabbit sera were pooled for further use.

Various samples were tested for the presence of antigens precipitable by anti-lipovitellin by loading them on to 1% agar gels (0.05 M barbital–HCl buffer, pH 7.2) for double diffusion analysis as described by Ouchterlony (1958). Diffusion was allowed to proceed for up to 48 hr at either room temperature or at 15°C.

Alternatively, samples of either sera or ovarian fractions were first run on 7% acrylamide gels as previously described, and then pairs of gels were placed parallel to each other, about 4 cm apart, in the bottom of a 10 cm square Petri dish. Warmed, buffered agar was poured over the gels so that it just covered them. After the agar had cooled, a 1.0 mm × 9.0 cm slit was made in the agar so that it was equidistant and parallel to the two acrylamide gels. Antiserum was added to the trough, and diffusion was allowed to proceed for 24–48 hr at either room temperature or at 15°C.

Agar from either of the diffusion methods was finally washed in several changes of 0.9% NaCl, stained by immersion in 0.5% Amido black in 7% acetic acid, and destained by washing in several changes of 7% acetic acid.

*EDTA; ethylenediaminetetracetic acid.

RESULTS

Life history observations

The mature female lobsters examined in this study alternated a molt with an oviposition, but on a variable schedule. Usually, molting occurred in one summer season with oviposition occurring in the next, but occasionally a molt was followed within a month or so with oviposition. A detailed description of the reproductive cycles for female *Homarus americanus* can be found in Aiken and Waddy (1976), and all the lobster reproductive patterns observed in the present study fit one of the patterns described in their review. In summary, females examined in this study were either undergoing their first round of vitellogenesis following a "puberty" molt (designated as pubertal and adult I) or were proceeding through their second vitellogenesis (thus adult II and adult III, depending on the timing of the molt). Adult III lobsters invariably followed the same seasonal molt-oviposition pattern described above, that is, a molt followed within a month by an oviposition, whereas the adult II lobsters followed the more predominant pattern of molt and oviposition in successive seasons.

Qualitative protein analysis

Hemolymph samples for electrophoretic analysis were taken from mature intermolt females during the summer months. An estimate of the vitellogenic state of the ovaries of these animals was made by calculating an ovarian factor (Table 1). Densitometric scans of the electrophoretic profiles of the serum samples from females with ovarian stage I to V inclusive exhibited 10–11 detectable protein bands with measured R_f values of 0.03–0.92. The method of electrophoretic analysis was reproducible; 40 randomly selected samples from the hundreds of samples analysed showed little variation in the relative migration of several stained components (Table 2).

The electrophoretic analysis of serum samples from females with vitellogenic ovaries (stages II to V inclusive) showed that a female specific protein (FSP) band (R_f 0.107) was present at highest concentration in the hemolymph of females with ovaries in stage III. This band was absent from male hemolymph, and was not detectable in the hemolymph of either immature females or females with ovaries in stage I (Fig. 1).

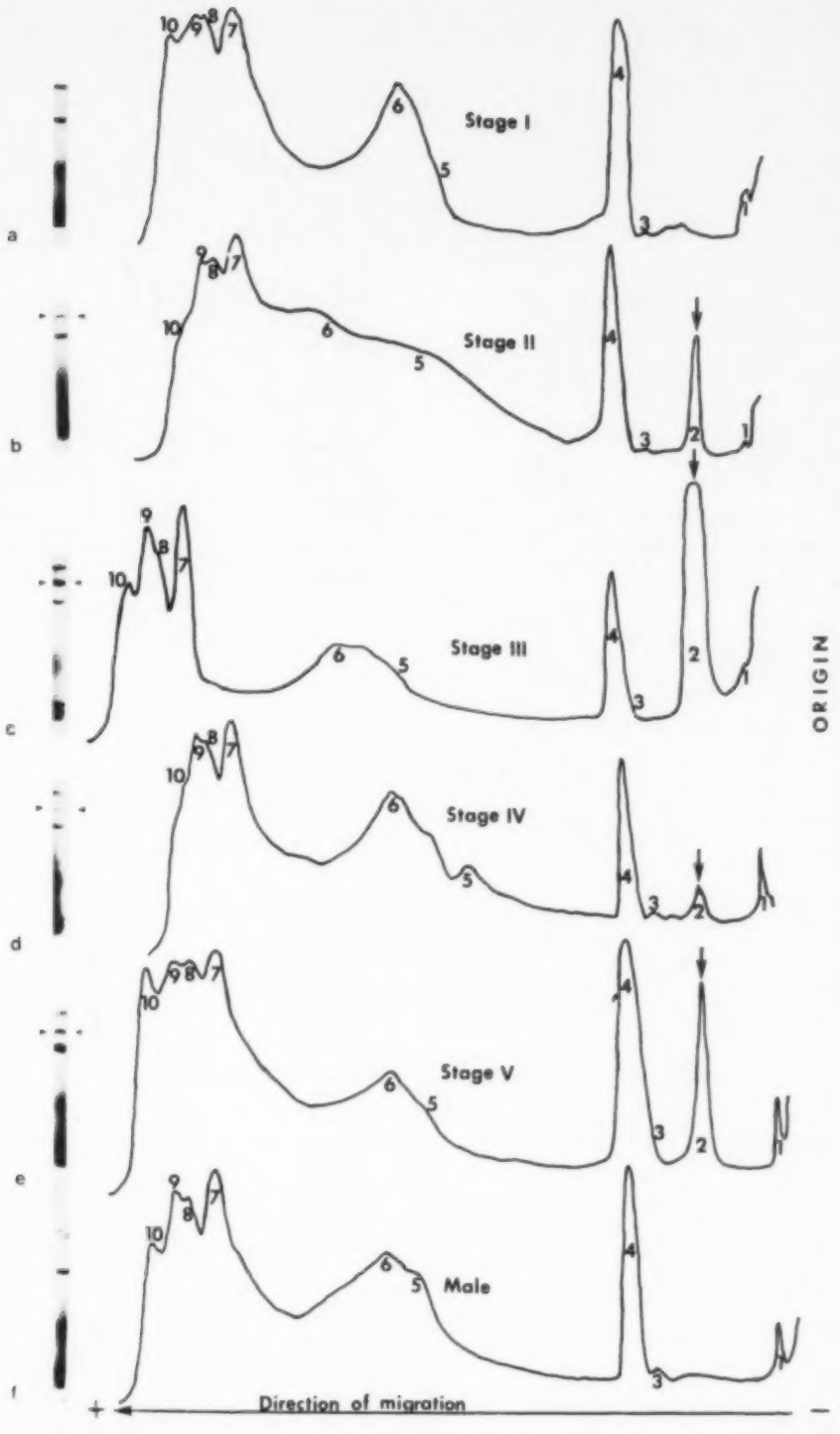


Fig. 1. Representative photographs and densitometer tracings of female (a–e) and male (f) serum proteins present after separation on 7% acrylamide gels at pH 8.8–9.0. Females exhibited ovarian stages from I–V as indicated (see Table 1 for description of stages). Arrows denote the female specific protein, where present, at an R_f value of 0.107.

Table 1. Ovary stages and descriptions for *Homarus americanus*

Ovarian stage*	Physiological stage	Description (*in situ*)	Range of ovarian factors (O_f) observed†
I	Immature	White, creamy	9–120
II	Post-oviposition	Yellow, flaccid	80–160
III	Vitellogenesis	Light green	127–178
IV	Vitellogenesis	Dark green	200–320
V	Mature, ovulation	Dark green	320–400

*Designations are similar to those used by Krouse (1973) for *Homarus americanus*, and Bomirski and Klek (1974) for *Rhithropanopeus harrisii* and *Crangon crangon*.

†Ovarian factor $(O_f) = \dfrac{[\text{ovary weight (mg)}] \times 10}{\text{carapace length (cm)}}$.

Table 2. Relative electrophoretic mobilities of some lobster proteins*

Protein	Source	Observed mean R_f ± SE
Female specific protein	Female serum	0.107 ± 0.012 ($N = 40$)
"Complex" protein†	Male and female serum	0.204 ± 0.071 ($N = 40$)
Hemocyanin (4–5 bands)†	Male and female serum	0.809 ± 0.039 to 0.918 ± 0.031 ($N = 40$)
Purified lipovitellin	Homogenate of mature ovary	0.106 ± 0.007 ($N = 5$)

*Gels used were 7% acrylamide at pH 8.8–9.0, run at 5–10°C.
†Identification after Fielder *et al.* (1971).

Electrophoretic preparations of crude and partially purified lipovitellin (Fig. 2) exhibited a major band with an R_f value of 0.106, identical to the R_f value of the hemolymph FSP found in vitellogenic females.

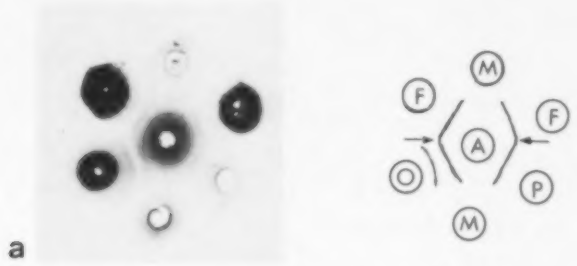
To confirm the identity of the hemolymph FSP and the lipovitellin fraction double immunodiffusion in agar was performed, using pooled preparations of rabbit anti-lipovitellin. The antibody formed single precipitin lines when tested against the original lipovitellin preparations, sera from females with ovaries in stages II to V inclusive, and crude ovarian homogenates (Fig. 3a). Precipitin arcs were confluent on

Fig. 2. Photographs and densitometer tracings of (a) a 20,000 *g* supernatant of a 0.5 M NaCl ovarian homogenate, and (b) purified lipovitellin, after electrophoresis on 7% acrylamide gels at pH 8.8–9.0. Both samples are 50 μg protein. The major bands (arrows) have R_f values of 0.106.

all plates tested, thus confirming the immunological identity of the serum FSP and lipovitellin. Male serum did not precipitate the anti-lipovitellin under the test conditions. When anti-lipovitellin was allowed to diffuse in agar toward electrophoretically separated samples of either female serum or ovarian homogenates, single precipitin arcs were seen adjacent to the R_f 0.107 position only (Fig. 3b). No other arcs were detectable, and, again, male serum did not precipitate the anti-lipovitellin under these conditions.

Hemolymph FSP and the molt cycle

We were further interested in the relationship between FSP concentration, molt stage, and ovarian stage. Non-ovigerous females were sampled throughout the year, with particular attention paid to the months of July and August, the period when vitel-



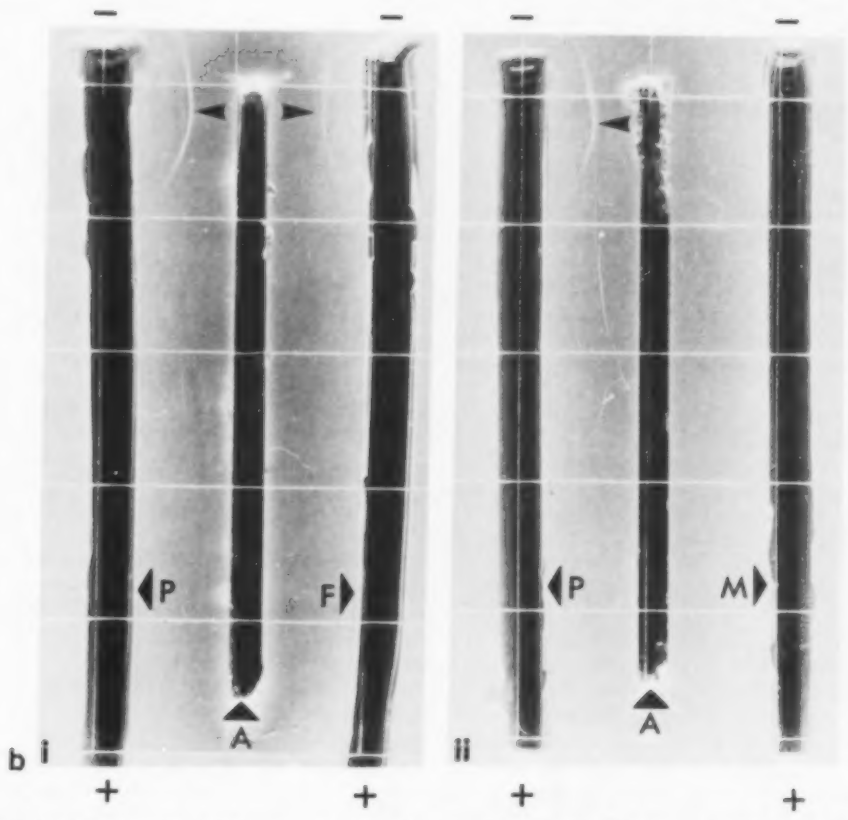


Fig. 3. (a) Photograph and diagram of a stained immunodiffusion slide. The arcs are confluent (arrows), suggesting identity of the proteins in wells F, P, and O. A = anti-lipovitellin; F = female serum previously shown to exhibit FSP on 7% acrylamide gels; M = male serum; O = crude ovarian homogenate supernatant; P = purified lipovitellin. (b) Diffusion (in agar) of anti-lipovitellin (A) against electrophoretically separated samples (in polyacrylamide gels, origin at top of figure) of purified lipovitellin (P), female serum with FSP (F, i), and male serum (M, ii). A single, identical precipitin arc is seen with each of the purified lipovitellin and the female serum, but the male serum shows no visible precipitin reaction.

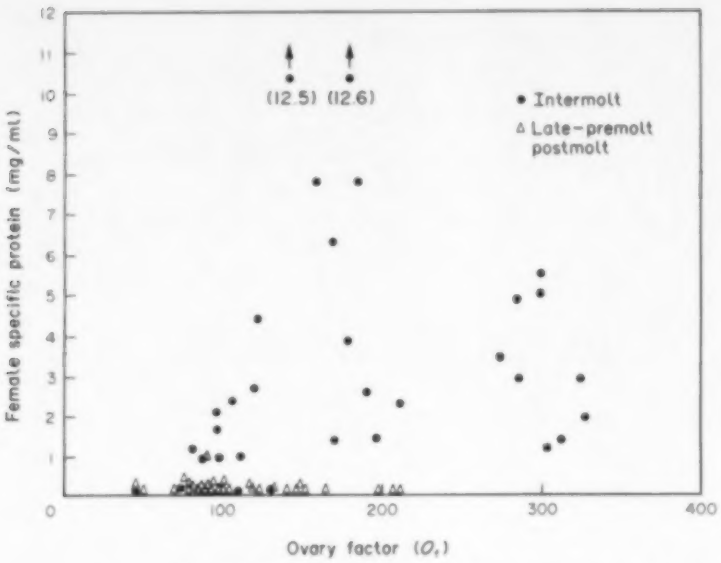


Fig. 4. Concentrations of serum FSP as a function of ovarian factor (O_f) in mature, non-ovigerous females during the July and August. Levels of FSP were determined from quantitation of serum samples separated on 7% acrylamide gels at pH 8.8–9.0. Each point represents a serum sample from a different lobster. The circles (●) represent samples taken from females in July–August in molt stages C to D_0 (intermolt to early premolt), whereas the open triangles (△) represent samples taken from females in molt stages D_1 (premolt) to stage B (postmolt).

logenesis is at its peak in the Northumberland Strait population of lobsters.

The results of extensive sampling are summarized in Fig. 4. Females in molt stages C_2 to D_0 (intermolt) showed variable concentrations of FSP, with a maximum occurring at ovarian stage III, that is, well before oviposition. Females in late premolt (stage D_1 or later) or postmolt (stage B) showed low to nil concentrations of FSP, although ovarian stages up to stage IV were observed. No mature (stage V) ovaries were observed in any of the late premolt group. In fact, an occasional premolt female was observed to be reabsorbing yolk from the oocytes, a condition which can be readily identified by the massive degeneration of most of the mature oocytes in the ovary, and the appearance of the released green yolk protein in the hemolymph. The levels of reabsorbed hemolymph protein is often so high as to give the lobster a dark-green or even black hemolymph.

Seasonal variation in hemolymph FSP levels

Sequential serum samples were obtained from several females throughout a complete calendar year in order to make observations on the seasonal fluctuations of circulating FSP. As expected, the FSP concentrations varied in accordance with the molting and oviposition patterns. Some representative patterns are shown in Fig. 5. Several trends emerged and can be summarized as follows: (a) FSP levels are always maximal *prior* to oviposition and are always low prior to molting (Fig. 5 a, b and c); (b) FSP levels are low (or even zero) in the winter months, when the water temperature is as low as 0°C (Fig. 5 a, b, c); (c) the levels of FSP show a good correlation to the vitellogenic condition of the oocytes; that is, if FSP is not present, there is no measurable oocyte growth (Fig. 5c). Reabsorption of yolk from the oocytes (Fig. 5d) causes a major deviation in FSP measurements but causes no alteration in the timing of the next molt (compare Fig. 5 a, b, c with Fig. 5d).

DISCUSSION

Our findings indicate that the concentrations of the female-specific protein (FSP) in the hemolymph of gravid females is modulated by the molt cycle and by seasonal variations in the water temperature, and is generally a clear indicator of the vitellogenic state of the oocytes. Thus, these results are in agreement with many other studies that have reported the occurrence of female-specific hemolymph proteins in the crustacean orders Amphipoda, Isopoda, as well as in the Decapoda (as reviewed by Adiyodi and Adiyodi, 1970; Croisille *et al.*, 1974). These investigations report that, as found in the present study, the concentrations of the FSP in the hemolymph is reported to increase as vitellogenesis in the oocytes becomes maximal.

The FSP in the Crustacea appears completely analogous to the blood-borne vitellogenins that are synthesized in the livers of birds (Schjeide *et al.*, 1963; Heald and McLachlin, 1965), amphibians (Redshaw and Follett, 1971; Wallace and Bergink, 1974), and by the fat bodies of insects (Telfer, 1965; Engelmann, 1969, 1970). Vitellogenins are transported to the ovary where they become incorporated, by micropinocytosis, into the oocyte as yolk. In fact, in the vertebrates and the insects, virtually all the yolk protein in a mature oocyte is accumulated in this way; for example, Wallace *et al.* (1972) estimate that in the amphibian *Xenopus laevis*, 99% of the yolk plate-

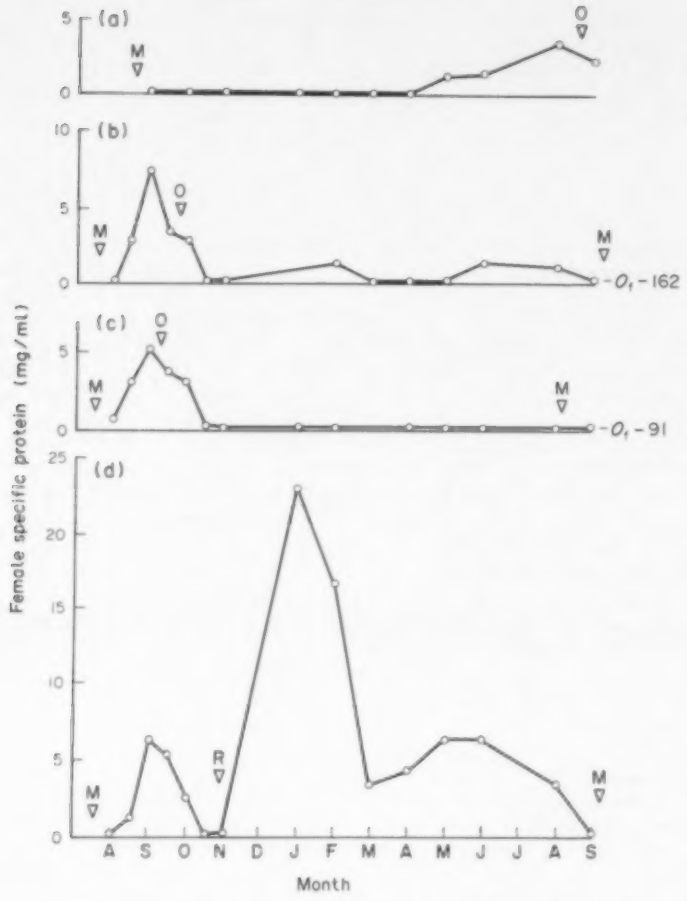


Fig. 5. Representative seasonal patterns of FSP levels in the serum of female lobsters sampled over an entire calendar year (August through the following September, inclusive). Each pattern graphed in (a) through (d) represents a single animal as followed throughout the year, and each point represents the measurement of the FSP concentration in a monthly serum sample. In (a) there was a molt (M) in one season, followed by a gradual increase in FSP levels and oviposition (O) the following summer. In (b) and (c) molt and oviposition both occurred in one summer, followed by only a molt the following summer. In (b), some vitellogenesis occurred ($O_t = 162$), whereas in (c) no vitellogenesis occurred ($O_t = 91$). Note that in (b), FSP was detectable in the spring of the second season, but this was not the case in (c). The typical pattern of reabsorption is shown in (d). Note that oviposition does not occur [compare with (b) and (c)], and that high levels of FSP persist throughout the winter months, but that this has no effect on the timing of the molt in the second season—[compare (b) and (c) with (d)].

let proteins phosvitin and lipovitellin are heterologous in origin.

The complete story with respect to the origin and fate of the crustacean FSP is less clear. Although FSP levels in the hemolymph of adult females seems to be a good index of vitellogenesis, with a maximum concentration occurring well prior to the maximum yolk accumulation in the oocytes, the central question as to the site of synthesis of FSP remains unclear. Two alternatives are proposed in the literature, and each alternative is supported by some, if incomplete, evidence.

The first alternative is that the FSP is synthesized, as are vertebrate and insect vitellogenins, outside the oocyte, and is transported there via the hemolymph (Kerr, 1969; Wolin *et al.*, 1973). The evidence for this point of view is considerable, although largely circumstantial, and is based on the consistent observations that the surface of the crustacean oocyte exhibits micropinocytotic pits and vesicles (Hinsch and Cone, 1969; Zerbib, 1973; Hinsch and Bennett, 1979; Schade and Shivers, 1980) and that the oocyte will take up exogenous proteins either from the hemolymph or from the external medium *in vitro* (Wolin *et al.*, 1973; Zerbib, 1977; Schade and Shivers, 1980). It is not known if the micropinocytosis is a receptor-mediated process (Goldstein *et al.*, 1979), nor has the site of synthesis of FSP been satisfactorily established.

The second alternative mechanism for the observed patterns of FSP in the hemolymph is that these proteins result only from reabsorption of yolk proteins that are synthesized in the oocyte. The evidence put forward for this alternative is both biochemical and ultrastructural. Lui *et al.* (1974) and Lui and O'Connor (1976) report that, in crayfish, each of the

three subunits of lipovitellin, the major yolk protein, are synthesized within the oocyte and conclude that extra-oocytic contributions to the total yolk protein seen in the mature oocyte are insignificant. Beams and Kessel (1963) and Ganion and Kessel (1972) concur, and describe the ultrastructural development of yolk granules in the extensive rough endoplasmic reticulum of the oocyte. Similarly, they conclude that micropinocytosis is present, but makes an insignificant contribution to the total yolk accumulated during vitellogenesis. Neither of these studies directly addresses the issue of the occurrence of female specific proteins present in the hemolymph, although Lui and O'Connor (1976) briefly state that these proteins could be accounted for by reabsorption of yolk, as has been described to occur in crayfish by Stephens (1952).

Our observations are best explained by the first alternative, that is, that FSP is in transit to the oocyte from an external source. The levels of hemolymph FSP that we observed were always highest well prior to the maximum accumulation of yolk in the oocytes, and the levels dropped off markedly prior to oviposition. This pattern is consistent with the idea that the FSP represents an externally synthesized protein that logically is found at the highest level in hemolymph during the period when the oocytes are accumulating the maximum amounts of yolk. If reabsorption were accounting for hemolymph FSP, then one might expect the maximum levels when the oocytes are the maximum size, that is, just prior to oviposition. In any case, our observations show that reabsorption is an abrupt and isolated event in lobsters which occurs usually some time after an otherwise normal period of vitellogenesis. The reabsorbed yolk is present in the hemolymph at huge concentrations (e.g. greater than 20 mg/ml) whereas FSP levels during vitellogenesis are considerably less (usually much less than 10 mg/ml). In other words, reabsorption is an event distinct from normal vitellogenesis.

Given the apparent contradiction in the evidence for yolk production in crustacean oocytes, it seems that the answer lies in a more complete assessment of the proportion of yolk that is synthesized in the oocyte, and the proportion that is imported from the hemolymph by micropinocytosis. There is clearly a component of the yolk that is synthesized inside the endoplasmic reticulum of the oocyte (Hinsch and Cone, 1969; Ganion and Kessel, 1972; Lui and O'Connor, 1976; Schade and Shivers, 1980), but it is equally clear that micropinocytosis is capable of taking up significant amounts of protein from the hemolymph (Schade and Shivers, 1980; Wolin *et al.*, 1973) and that micropinocytosis can be amplified by an appropriate hormonal signal (Hinsch and Bennett, 1979). Thus, that the total quantity of crustacean yolk protein is accumulated as a net result of both intraoocytic and extraoocytic syntheses seems certain; however, the relative contribution of each needs to be better defined.

Our detailed observations on individual lobsters over two full reproductive seasons allowed us to establish unequivocally that molt and oviposition are alternated on a variable schedule. This alternation is not surprising in that both molt and oviposition make major demands on metabolic reserves. But it is important to note that both molt and oviposition continue to occur in most reproductively mature crustaceans, thus separating the Crustacea from insects, to which they are often compared. Mature female insects do not molt, with the exception of the Thysanura (Rohdendorf and Watson, 1969). Thus the separation of molting and reproduction is temporally distinct, as are, presumably, whatever control mechanisms for the two processes that are at work.

In the Crustacea, on the other hand, the control mechanisms for molting and reproduction are more closely interwoven, and thus more difficult to analyze in isolation. That there is some kind of hierarchy between the two processes seems axiomatic, since an inopportune molt, that is, immediately after oviposition, would result in a loss of eggs on the cast exoskeleton. Our results indicate that in the period from late premolt (stages D_1 to D_3) to postmolt (stage B), the hemolymph FSP levels are low to zero in non-ovigerous females, although the ovaries in these same animals are up to stage III in development. Thus, it appears that premolt events have suppressed vitellogenesis, as judged by the hemolymph titer of FSP. In addition, late premolt lobsters with mature ovaries will reabsorb yolk rather than oviposit. These two observations, taken together, lead to the conclusion that premolt events take precedence over, and may disrupt events leading to normal oviposition. A similar hierarchy exists in the Thysanuran, *Lepismodes inquilinus*, one of this order of exceptional insects that continues to molt and grow after reaching sexual maturity. In this species, the molting cycle overrides reproductive activity, and artificially induced molting will cause reabsorption of yolk from the oocytes of mature ovaries (Rhodendorf and Watson, 1969). It seems that premolt events and oviposition are incompatible. A similar conclusion can be derived from an experiment in which lobsters (*Homarus americanus*) were subjected to 6 summer months in an artificially imposed 9-month year (Aiken and Waddy, 1976). The mature ovary was reabsorbed only when the molt and reproductive cycles coincided such that molt would occur shortly after oviposition. In other words, it appears that the physiology of late premolt cannot include the final vitellogenesis (at least past stage III) and other preparations leading up to oviposition.

In conclusion, we feel that the data on hemolymph FSP that are presented here provide a reliable index of vitellogenesis, and, could become a useful tool in further studies of lobster reproductive physiology.

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PINEAL MELATONIN IN HIBERNATING AND AROUSED GOLDEN HAMSTERS (*MESOCRICETUS AURATUS*)

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Abstract—1. Daily changes of pineal melatonin content were determined in warm-adapted nonhibernating and cold-adapted hibernating golden hamsters (*Mesocricetus auratus*).

2. Pineal melatonin in nonhibernating golden hamsters showed marked daily rhythm with the night values about 20 times higher than the daytime ones.

3. In hamsters hibernating for 2 and 3 days the melatonin rhythm was abolished, since no increase of pineal melatonin over basal levels occurred throughout 24 hr period.

4. After arousal from hibernation melatonin increased rapidly regardless whether the hamsters were provoked to arousal during day or night.

INTRODUCTION

Pineal melatonin in the golden hamster, *Mesocricetus auratus*, shows marked circadian rhythm with night values more than 10 times higher than the daytime ones (Tamarkin *et al.*, 1979). The rhythm is truly circadian since it persists in constant darkness; it is, however, inhibited by light (Tamarkin *et al.*, 1979, 1980). The melatonin rhythm in the golden hamster may be driven by the rhythm in *N*-acetyltransferase (NAT) activity (Vaněček and Illnerová, 1982), the enzyme forming the melatonin precursor *N*-acetylserotonin, similarly as in the rat, *Rattus norvegicus* and the Djungarian hamster, *Phodopus sungorus* (Klein and Weller, 1970; Illnerová *et al.*, 1983). In rats, the NAT rhythm is generated by daily changes in the turnover and presumably in the release of norepinephrine (NE) from sympathetic fibres innervating the pineal from superior cervical ganglia (SGC) (Brownstein and Axelrod, 1974; Klein *et al.*, 1971). NE released at night interacts with the beta-adrenergic receptors on the pineal cell membrane and through cAMP system induces NAT increase (Klein and Berg, 1970). The rhythm in NAT activity and thus also in pineal melatonin in rats are generated from suprachiasmatic nuclei (SCN) of the hypothalamus, the presumed circadian pacemaker in mammals (Klein and Moore, 1979; Rusak and Zucker, 1979).

Melatonin was shown to influence hibernation in several species. Chronic melatonin administration increased the incidence of spontaneous daily torpor in *Peromyscus leucopus*, prolonged the mean duration of hibernation in *Spermophilus lateralis* and increased frequency of hibernation in *Mesocricetus auratus* kept in constant light (Lynch *et al.*, 1978; Palmer and Riedesel 1970; Janský *et al.*, 1981). On the other hand, melatonin had no effect on hibernation in *M. auratus* and *S. tridecemlineatus* kept in darkness (Janský *et al.*, 1981; Sinnamon and Pivorun, 1981). Despite the role melatonin might have in hibernation, nothing is known about melatonin synthesis during hibernation. We therefore, determined pineal melatonin in golden hamsters during hibernation and immediately after arousal.

MATERIALS AND METHODS

Thirty-day-old male golden hamsters were maintained under short artificial photoperiods L:D 8:16 (lights on from 07:00 to 15:00 hr) and fed corn and carrots for at least 11 weeks before decapitation. Animals killed in darkness were exposed to a dim red light for less than 1 min. The pineal glands were dissected and stored in a Petri dish on solid CO_2 for melatonin determination. Within two days single pineals were homogenized in 100 μl of 0.05 M phosphate buffer pH 7; the homogenate was diluted with 500 μl of the same buffer and extracted with 1 ml of methylene chloride. The organic phase was washed twice with 0.2 ml of 0.1 M NaOH and evaporated to dryness. Melatonin was estimated in the dry residue by the RIA developed by Arendt *et al.* (1975), as modified by Wetterberg *et al.* (1978). The amount of melatonin was expressed as pg per one pineal gland, the limits of detection of the assay were 6 pg per tube. The intra- and inter-assay variations were 8.1% (for 50 pg, $N = 5$) and 11.3% (for 50 pg, $N = 4$) respectively. Kits for melatonin were purchased from Kabi diagnostica, Stockholm, Sweden. Statistical evaluations were made by Student's t-test.

Experiment 1

The hamsters were caged either four per cage at a temperature of 25 ± 1°C or singly in a cold room in a deep basement where temperature gradually increased in the course of a year from 5.5°C to 13°C. After 6 weeks of cold exposure the animals began to hibernate in a typical interrupted fashion (Janský *et al.*, 1981): hibernation bouts lasting 1–3 days were followed by 1–2 days arousal. Within 11 weeks 73% of cold exposed hamsters entered regular hibernation, 13% of animals did not hibernate at all and 14% exhibited occasional hibernation bouts. The torpor was monitored by the "sawdust on the back" technique and checked daily at 11:00 hr. Hamsters which entered torpor later than at 11:00 hr were considered to be just entering a hibernation bout (H–0) until the next checking. From

11:00 hr of the following day, if the torpor still persisted, they were considered to be in the first day of a hibernation bout (H-1) etc. Warm-exposed and hibernating cold-exposed hamsters were both killed after 11–13 weeks of exposure to the described conditions.

Experiment 2

The hamsters were caged singly in a cold room, where temperature ranged from 8°C to 14°C. After about 4 months, the animals hibernating previously for 2 days were transferred at 8:30, 16:30 or 0:30 hr to a temperature of 28–30°C and shaken to provoke their arousal. All manipulations were done under dim red light and thereafter the animals were kept in complete darkness until they were killed. At the time of the sacrifice all animals were active.

RESULTS

The melatonin content in the pineals of hamsters kept at a temperature of 25°C showed a marked daily rhythm; the night levels were about 20 times higher than the day levels (Fig. 1A). The melatonin content was 64 ± 21 pg per pineal at 15:00 hr and 1267 ± 174 pg per pineal at 01:30 hr. The melatonin concentration started to increase at about 22:00 hr and declined at about 06:15 hr.

In hamsters hibernating for at least 2 days, no rhythm in pineal melatonin was observed (Fig. 1B). The melatonin content ranged from 37 ± 9 pg per pineal to 73 ± 23 pg per pineal. However, the rhythm in pineal melatonin content persisted in the beginning of each hibernation bout (Table 1). In hamsters entering the hibernation bout (H-0) the melatonin concentration at 05:15 hr was about eight times higher than the baseline levels at 08:00 hr, and the values were similar to those in nonhibernating hamsters killed at the same time. In animals on the first day of hibernation (H-1) the melatonin concentration at 20:30 hr was still about 2.5 higher than at 08:00 hr. The melatonin rhythm is thus not abolished immediately after entry of animals into a hibernation bout, but it rather takes several hours before the rhythm disappears.

Table 1. Pineal melatonin content in hamsters in early stages of hibernation bouts

Time	*N*	Hibernating stage	Melatonin (pg/pineal)
05:15	3	H-0	465 ± 83*
08:00	3	H-0	55 ± 11
20:30	3	H-1	194 ± 31*
08:00	4	H-1	75 ± 24

*$P \leq 0.05$ vs the corresponding value at 08:00 hr. Values are the means ± SEM.

After arousal pineal melatonin increased rapidly regardless of whether hamsters were provoked to arousal at 08:30, 16:30 or 00:30 hr (Fig. 2). Within 12–16 hr after arousal the pineal melatonin content in all these groups reached similar levels as in nonhibernating warm-adapted hamsters during night. However, in hamsters aroused at 00:30 hr and 16:30 hr melatonin concentration increased slightly more rapidly than in the animals aroused at 08:30 hr.

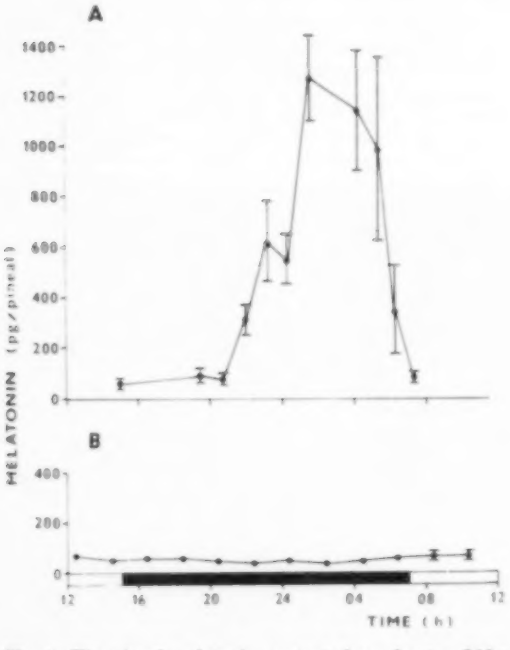

Fig. 1. The pineal melatonin content throughout a 24 hr period in warm-adapted (A) and hibernating cold-adapted (B) golden hamsters kept under L:D 8:16 photoperiods. Hibernating hamsters were killed on days 2 or 3 of a hibernation bout. The black bar represents the dark portion of the light–dark cycle. Values are the means ± SEM from four animals. SEM is omitted when its value is smaller than the radius of the point.

DISCUSSION

The rhythm in pineal melatonin concentration in warm-adapted hamsters was similar to that reported by Rollag *et al.* (1980) for hamsters exposed to L:D

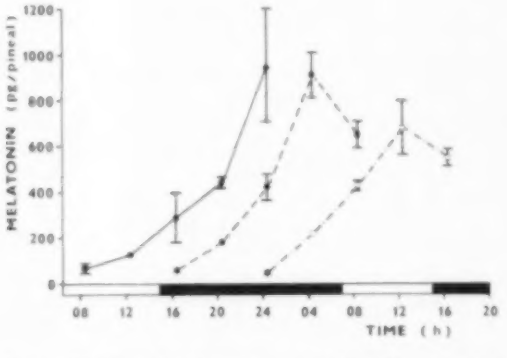

Fig. 2. The pineal melatonin content in golden hamsters within 16 hr after arousal from hibernation bout. Animals kept in cold under L:D 8:16 photoperiods and hibernating previously for two days were provoked to arousal at either 08:30 hr —●—, 16:30 hr —■—, or 00:30 hr (—·□·—), then they were kept in darkness and killed either 4, 8, 12, or 16 hr later. ★ Values of hibernating hamsters killed at 08:30, 16:30 and 0:30 hr redrawn from Fig. 1. Values are the means ± SEM from 3–4 animals. SEM is omitted when its value is smaller than the radius of the point. The black bar represents the dark portion of the previous light–dark cycle.

08:16 for 10 weeks. The pineal melatonin in hibernating mammals has not been studied so far, although it was shown that melatonin might influence hibernation in several species (Lynch *et al.*, 1978; Palmer and Riedesel, 1976; Janský *et al.*, 1981). We observed that the rhythm in pineal melatonin was abolished in golden hamsters in days 2 and 3 of hibernation bout. Similarly, Vivien-Roels *et al.* (1979) did not find any melatonin rhythm in the turtle *Testudo hermanni* during its winter torpidity. The abolishment of pineal melatonin rhythm might be due either to (i) arrest of circadian pacemaker; (ii) to inhibition of melatonin synthesis in the pineal gland by cold; or (iii) to inhibition of neural transmission from pacemaker to the pineal gland.

(i) It is possible that the circadian pacemaker might be arrested at a certain phase when body temperature in hibernating hamster decreases below certain limit and then be released again after arousal when tissue temperature increases. If we presume that the pacemaker is arrested at the phase close to light–dark transition, this hypothesis could explain the inhibition of melatonin rhythm during hibernation as well as the rapid increase of melatonin after arousal. However, literary data do not support the hypothesis. It was reported that circadian pacemaker was not inhibited in bat, *Eptesicus fuscus*, and in garden dormouse, *Eliomys quercinus*, hibernating at a temperature below 10°C (Menaker, 1959; Saint Girons, 1965).

(ii) Alternatively, it is possible that melatonin synthesis in the pineal might be inhibited by low tissue temperature. However, in hamsters which entered hibernation before 11:00 hr (H-1, Table 1) pineal melatonin at 20:30 hr was 2.5 times higher than the daytime basal levels. Thus at least 9 hr after entry to hibernation when the body temperature should be already close to ambient temperature (Lyman and Chatfield, 1955; Janský *et al.*, 1983), melatonin synthesis in the pineal was significantly increased above basal levels. Moreover, if the daily rhythm in NE release from pineal nerves would continue and only melatonin synthesis would be inhibited during hibernation, then after arousal the melatonin would increase with a various lag phase depending on daytime of arousal. However, melatonin increased immediately regardless whether arousal occurred during day or night.

(iii) Therefore a more plausible hypothesis is that neural transmission from a circadian pacemaker to the pineal gland may be inhibited during hibernation. It is presumed that activity sympathetic system is reduced during hibernation and stimulated during arousal and melatonin rhythm is probably driven by the rhythmic release of NE from sympathetic nerves (Lyman and Chatfield, 1955; Lyman and O'Brien, 1963; Hudson and Wang, 1979; Twente and Twente, 1978). Hence, the abolishment of the melatonin rhythm during hibernation might be due to the inhibition of the sympathetic system, namely to reduction of NE release from the nerve endings in the pineal. After arousal, catecholamines released due to activation of sympathetic-adrenal system (Lyman and O'Brien, 1963; Hudson and Wang, 1979) might induce a rapid increase of melatonin synthesis. However, before a definite explanation might be given, it is necessary to follow the melatonin rhythm after arousal for a longer period.

In conclusion, we observed that the melatonin rhythm in golden hamsters was abolished during hibernation and that pineal melatonin increased rapidly after arousal.

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DRINKING OF FRESH WATER BUT NOT SEAWATER BY THE ESTUARINE CROCODILE (*CROCODYLUS POROSUS*)

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Abstract—1. The roles of drinking and integumental exchange in water balance of unfed estuarine crocodiles, *Crocodylus porosus*, have been examined in both seawater and fresh water.

2. In seawater, *C. porosus* avoids drinking despite the loss of substantial amounts of water across the integument. Water loss across the buccal mucosa accounts for 60% of the total.

3. In fresh water, *C. porosus* experiences a small net uptake across the integument which is insufficient to compensate for urinary water losses. Water balance is achieved through drinking.

4. Together with data from other field and laboratory studies, the evidence supports the view that *C. porosus* in seawater may drink fresh water facultatively but does not depend on drinking for maintenance of hydromineral balance.

5. Review of the available information on drinking in other aquatic reptiles suggests that the results for *C. porosus* should not be extrapolated to the Crocodylia as a whole. It appears that further study of crocodilians and other aquatic reptiles will reveal a wider range of drinking strategies than has been recognized previously.

INTRODUCTION

Drinking of water, either salt or fresh, cannot be overlooked as a potentially important component of the osmoregulatory strategy of any aquatic reptile. It may allow compensation for evaporative, osmotic or excretory water losses in situations where integumental uptake or the water intake in food is insufficient to maintain "normal" hydration status. Yet review of the literature reveals that relatively little attention has been paid to the role of drinking *vis à vis* other routes of uptake in either fresh water or estuarine/marine reptiles. A number of studies have provided indirect evidence that salt water reptiles such as the sea snake *Pelamis platurus*, the estuarine turtle *Malaclemys terrapin* and the mangrove snakes, *Nerodia fasciata clarki*, *N. f. compressicauda* and *N. valida*, do not normally drink seawater (SW) (Dunson and Robinson, 1976; Robinson and Dunson, 1976; Dunson, 1978, 1979). However, no direct experimental tests of drinking behavior in any of these reptiles have been reported to date. The situation is little better for freshwater (FW) reptiles in which the only direct tests of drinking are those of Bentley and Schmidt-Nielsen (1965), Cloudsley-Thompson (1968, 1969) and Diefenbach (1973) on the crocodilians, *Caiman crocodilus* and *Crocodylus niloticus*, and a study of water balance in the turtles *Chrysemys scripta* and *Trionyx spiniferus* by Bentley and Schmidt-Nielsen (1970).

Despite the three studies of water balance in crocodilians, it is still not clear whether their evaporative and excretory water losses in FW are compensated primarily by drinking or by integumental uptake. Cott (1961) first suggested that basking crocodiles might replace evaporative water losses by immersing the hindbody or tail in the water and absorbing water through the integument. Cloudsley-Thompson (1968, 1969) inferred from experiments with a small number of Nile crocodiles, *C. niloticus*, that integumental water absorption was negligible, rehydration being achieved normally by drinking—a conclusion supported by Diefenbach's (1973) work on *C. niloticus* and *Ca. crocodilus*. However, neither of these studies included adequate controls for respiratory water loss so a small component of integumental uptake might have passed unnoticed. Bentley and Schmidt-Nielsen (1965), on the other hand, determined that uptake across the skin of *Ca. crocodilus* accounted for 70% of the total influx in FW. This result has been queried, however, on the basis of doubts about the reliability of the phenol-red technique used for measuring drinking rates (Diefenbach, 1973). As for crocodilians in salt water, even less is known. Some anecdotal reports suggest that the estuarine crocodile, *Crocodylus porosus*, discriminates salt from fresh water. For example, a 5 m *C. porosus* held in SW at the Green Island Aquarium in northern Queensland is provided periodically with FW from a hose and will actively seek it out, open its mouth under the stream of water and drink (H. Messel, personal communication). Such observations do not, however, indicate that *C. porosus* does not drink SW, nor do they reveal anything about the contribution of drinking to the overall water budget.

There remains, therefore, considerable uncertainty as to the relative importance of oral and integumental water uptake in crocodilians. As part of a comprehensive investigation of the osmoregulatory physiology of the estuarine crocodile, *C. porosus*, in both fresh and salt water (Taplin, 1982), it was necessary to resolve this uncertainty and to determine also:

(a) whether this species can osmoregulate by drinking seawater (SW) and excreting the excess salts

through the lingual salt glands described by Taplin and Grigg (1981); and

(b) whether it is able to discriminate salt from freshwater and, therefore, has the ability to recognize and make use of localized FW springs in saline estuaries.

In the present study, the partitioning of net water flux between integumental and oral routes of uptake or loss has been determined for unfed *C. porosus* in both FW and SW. The results show clearly that integumental influx in FW plays only a minor role in the overall water budget, the majority of influx occurring through drinking. In SW, on the other hand, *C. porosus* does not drink but suffers substantial osmotic water loss which must normally be compensated by feeding. The available evidence does not, however, allow the conclusion that integumental uptake is necessarily negligible in all crocodilians nor that avoidance of drinking in SW is a universal phenomenon among estuarine/marine reptiles.

MATERIALS AND METHODS

It should be noted at the outset that the application of isotopic techniques, using tritiated water, to tests of net water uptake through drinking or integumental exchange is not practical for the simple reason that the small net flows of interest are swamped by a very large diffusional exchange of water across the integument. Detailed water budgets for *C. porosus* in both FW and SW, derived from THO studies, are discussed in detail by Taplin (in prep., b).

Drinking rates were measured gravimetrically in hatchling crocodiles [80–150 g body weight (BW)] reared in FW in our Sydney laboratory. Animals were starved in FW for 3–4 days and their cloacas drained of urine. Each was then dried carefully, using towels and a jet of air played over its skin and buccal membranes, before being weighed to 0.1 g. They were exposed to SW or FW in 50 l plastic bins sealed with plastic food wrap to maintain an essentially saturated atmosphere. Relative humidity in the containers was measured at 96–98% relative to saturated $K_2Cr_2O_7$ and NaCl standards (Winston and Bates, 1960) using porous pot humidity probes. The entire apparatus was maintained at 25 ± 0.5°C in a constant temperature room.

A rubber finger cot was glued over the cloaca of each crocodile using "Supa-Glue" and the animals restrained individually on perspex boards using PVC tape. Each board was then immersed at an angle of roughly 45° from the horizontal so that the crocodile was immersed up to its neck and able to dip its mouth into the water to drink. Half of the animals were prevented from drinking by the simple expedient of strapping wooden blocks to the boards, thus preventing the animals from tilting their heads into the water. Crocodiles were removed after 48 hr exposure, dried, weighed once more and the volume of urine in the collection sac measured.

Respiratory water loss was estimated at 0.05 ml/day based on a total water loss rate in *Caiman* of 65 μg/(cm^2 hr mm Hg saturation deficit) and a respiratory water loss 25% of that total (Schmidt-Nielsen, 1969). Catabolic C loss was estimated at 0.04 g/day based on data of Bennett and Dawson (1976). Both of these components were sufficiently small relative to the weighing errors to be ignored. Net uptake or loss of water was, therefore, calculated according to the balance equation:

$$\Delta BW = \text{drinking} + \text{net integumental uptake} - \text{urination}.$$

Sixteen crocodiles were used in four replicate experiments in which one crocodile was assigned to each of the treatment combinations; SW–"drinking", SW–"non-drinking", FW–"drinking", FW–"non-drinking". Two of the crocodiles in SW dislodged their urine collection bags so a separate determination was made on four crocodiles in SW; two "drinking" and two "non-drinking". Data for all experiments were combined and four replicates of each treatment selected at random for analysis by balanced 2-factor ANOVA.

To test the possibility that restraint might have inhibited drinking in crocodiles exposed to SW, a further 10 animals (65–88 g BW) were tested free-swimming in SW. Attempts to attach urine collection sacs were unsuccessful as they were invariably dislodged. Nor was cloacal cannulation used because of the likelihood of stimulating artificially high urination rates where the cloaca was drained continually. Instead, cloacas were sealed using "Supa-Glue" and experiments run over as brief a period as possible (48 hr) so that any inhibition of drinking attributable to preventing urination would be minimized. Half of the crocodiles in these experiments had their mouths taped shut with PVC tape and sealed at the edges using Silastic Type 5145 Silicone sealant. In other respects the experimental regime was the same as in the earlier experiment except that all crocodiles were treated simultaneously in one tank.

Both of these techniques suffered from the obvious disadvantage that the buccal cavities of "non-drinking" crocodiles were out of contact with water. Thus, osmotic uptake of water across the buccal membranes in FW could be confounded with uptake due to drinking. To test this possibility a group of 10 hatchlings was dehydrated in SW to about 85% of their initial BW. Unfed *C. porosus* of this size in SW lose weight linearly over time at rates of 1.5–2%/day (Taplin, in prep., b). They were then returned to FW and their rate of recovery monitored over the following three days by repetitive measurement of BW.

Surface area (SA) of crocodiles was calculated from the formula derived by Dunson (1982) for *C. acutus*; SA (cm^2) $= 11.47 \times BW^{0.637}$ which, for animals <1 kg or so, gives good agreement with the equation used by Bentley and Schmidt-Nielsen (1965). Unless otherwise specified, values are expressed as $\bar{X} \pm SE(N)$ where SE = standard error of the mean.

RESULTS

The estuarine crocodiles used in this study drank fresh water when allowed to but very obviously avoided drinking seawater. Restrained hatchlings in SW were in negative water balance, losing water at essentially identical rates regardless of whether they were allowed to drink or not (Table 1). Rates of water uptake in fresh water, however, were some six times higher in "drinkers" than in "non-drinkers" (Table 1). Analysis of variance indicates that the difference between "drinkers" and "non-drinkers" in FW is highly significant ($0.001 < P < 0.002$) while the difference in SW is not significant at even the 50% significance level.

Unrestrained hatchlings in SW produced very similar results except that, with complete isolation of the buccal cavity from the surrounding water, "drinkers" and "non-drinkers" showed significantly different rates of water loss. Hatchlings with sealed mouths lost water at $44.7 \pm 4.04(5)$ μl/100 g hr compared with $74.4 \pm 3.32(5)$ μl/100 g hr in crocodiles with their buccal membranes exposed to SW. Evidently, the thinly keratinized buccal epithelia are very much more permeable to water than the rest of the integument.

The higher permeability of the buccal mucosa raised the possibility that water uptake in FW-exposed crocodiles without mouth seals could be due

Table 1. Water balance in hatchling *C. porosus* exposed to SW or FW at 25°C ($\bar{X} \pm$ SE)

	Seawater		Fresh water	
	Drinking	Non-drinking	Drinking	Non-drinking
N	4	4	4	4
BW(g)	86.1 ± 2.34	103.3 ± 3.59	93.1 ± 5.68	103.0 ± 16.57
Net water flux (μl/100 g/hr)	− 20.5 ± 4.02	− 21.6 ± 2.59	+ 23.6 ± 2.97	+ 4.6 ± 3.11
Urine production (μl/100 g/hr)	36.4 ± 15.46	35.8 ± 8.98	51.2 ± 9.88	49.4 ± 15.86
Net integumental flux (μl/100 cm^2/hr)	− 10.2 ± 1.89*	− 9.6 ± 0.91	—	+ 2.2 ± 1.54

*This value has been calculated on the assumption that all net water flux in this group is attributable to integumental exchange.

to osmotic uptake rather than drinking. However, the time course of weight changes in crocodiles exposed to SW and returned to FW demonstrates clearly that this is not the case (Fig. 1). Hatchlings lost weight in SW at a mean rate of 2.2% of their initial BW per day (%BW$_i$/d) but recovered to 96% of their initial weight within 1 hr of their return to FW and to 98% within 4 hr. Given that the osmotic pressure gradient between SW and body fluids is opposite to and roughly twice as high as that between FW and body fluids, the rapid recovery in FW can only reasonably be attributed to drinking. The dramatic weight increase over the first hour or so in FW required the uptake of 7–8 ml of water per animal (some 10% of BW$_i$) and, not surprisingly, these hatchlings had very distended abdomens suggesting that their stomachs were, indeed, full of water.

DISCUSSION

For *C. porosus* in fresh water, and probably other crocodilians also, drinking is evidently an important component of the overall osmoregulatory strategy. Osmotic uptake of water across the integument in FW accounts for only a small fraction of the total net uptake and is insufficient to compensate for the large urinary water loss (Table 1). This finding is at odds with those of both Bentley and Schmidt-Nielsen (1965), Cloudsley-Thompson (1968, 1969) and Diefenbach (1973). Neither Cloudsley-Thompson nor Diefenbach found evidence of integumental water uptake in *C. niloticus* or *Ca. crocodilus* but they failed to control effectively for evaporative water loss so may well have missed a small component of uptake by this route. Bentley and Schmidt-Nielsen, on the other hand, showed that *Caiman* appears to take up some 66 μl H_2O/100 cm^2 hr across the integument in FW compared with only 2 μl/100 cm^2 hr in *C. porosus* (Table 1). The large difference could simply reflect some deficiency in the phenol-red method of measuring drinking rates but could equally reflect a real difference in integumental permeability between the two species. The latter possibility receives some support from a consideration of Bentley and Schmidt-Nielsen's (1970) study of water balance in the FW turtles, *Trionyx spiniferus* and *Chrysemys scripta*.

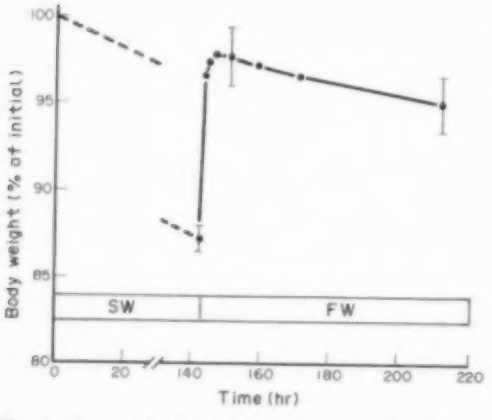

Fig. 1. Changes in BW of hatchling *Crocodylus porosus* exposed to seawater for 140 hr and then transferred directly into fresh water. The broken line represents the average rate of fall in BW over the 140 hr of exposure. Taplin (in prep., b) has shown that weight loss in unfed *C. porosus* in seawater is essentially linear over time in both hatchling and juvenile animals (0.1–10 kg BW). Error bars are SEM.

Drinking in FW reptiles must inevitably increase urinary electrolyte losses while allowing little or no uptake of Na from the gut and must, therefore, aggravate the problems of Na and water balance in an electrolyte-depleted medium. It seems likely, then, that the high urinary water loss measured in *C. porosus* (Table 1) is obligatory and reflects the necessity to excrete waste products of metabolism. The low integumental water permeability in *C. porosus*, $P_{osm} = 0.017$ μl/(100 cm^2 hr mOsm), precludes compensation by osmotic uptake so active drinking is necessary. *Trionyx*, in contrast, has a very permeable integument ($P_{osm} = 0.37$ μl/(100 cm^2 hr mOsm) (calculated from Bentley and Schmidt-Nielsen, 1970) and drinks only 8 μl/100 g hr despite a urinary loss of 180 μl/100 g hr. *Chrysemys*, on the other hand, shows much lower integumental uptake [$P_{osm} = 0.08$ μl/(100 cm^2 hr mOsm)] but a similar overall turnover rate to *Trionyx* and, in keeping with the necessity to maintain water balance, drinks 130 μl/100 g hr. Thus, where integumental permeability is low, drinking of FW may be necessary to replenish excretory water loss. The apparently small contribution of drinking to total water uptake in *Caiman* may, therefore, be real and reflect a higher integumental permeability than found in *C. porosus*.

The occurrence of drinking in freshwater crocodilians is of interest because, as noted above, it must inevitably tend to aggravate their salt balance problems and is presumably necessary for excretion of waste products, particularly nitrogen. The principal urinary nitrogenous excretory product of *C. porosus* in FW is ammonia, with only low levels of urea, urates and creatinine (Grigg, 1981). As *C. porosus*

appears well able to reduce urinary water loss in SW by excreting insoluble urates, its excretion of ammonia in FW must convey some advantages which outweigh the cost in extra electrolyte losses. Apart from the small saving in free energy consequent on a shift from urate to NH_3 excretion, the most obvious advantage lies in the possibility of linkage between NH_4HCO_3 excretion and NaCl reabsorption in the kidney/cloacal complex. NH_4HCO_3 excretion, while requiring greater water losses than urate excretion, may facilitate NaCl reabsorption from the urine (Coulson and Hernandez, 1970; Schmidt-Nielsen and Skadhauge, 1967). This possibility must be reconciled, however, with the facts that, in fasting alligators, at least 90% of the NaCl reabsorption from glomerular filtrate takes place independently of any NH^{4+}/Na^{+} exchange mechanism (Coulson and Hernandez, 1970) and that, in SW-acclimatized *C. porosus*, the Na concentration in the urine is no higher than in FW-acclimatized crocodiles despite a marked decline in NH_3 excretion (Grigg, 1981; Taplin, 1982). Further investigation of the linkage between nitrogen excretion and salt conservation in SW- and FW-adapted reptiles would be of great value in understanding the drinking behaviour of both crocodilians and turtles.

The evidence that *C. porosus* avoids drinking SW is consistent with the observations of Taplin (in prep., b) that unfed *C. porosus* in SW dehydrate more or less rapidly and will die eventually if not allowed access to FW. Yet, despite their obvious ability to discriminate salt from fresh water, evidence from the field demonstrates that the availability of FW is not necessary for survival in SW. On the Liverpool/Tomkinson River System, focus for much of our physiological work on *C. porosus* in Australia (Grigg *et al.*, 1980; Grigg, 1981; Taplin and Grigg, 1981), prolonged rain-free periods of 2–4 months duration are common and sources of FW in downstream saline habitats are limited to a few discrete and widely scattered springs or lagoons (Taplin, 1982; in prep., a). Grigg *et al.* (1980) showed that *C. porosus* hatchlings (160–250 g BW) in hypersaline salt water do not appear to use the FW springs potentially accessible to them, even when FW lies close to or within their apparent home range. Furthermore, many hatchlings and larger crocodiles can be found consistently in sites far removed from FW and would have to be making repeated journeys of 10–20 km by river or overland to account for their repeated captures at these remote sites. Though this might be feasible for large crocodiles, it is out of the question for hatchlings. Neverthless, both hatchlings and larger crocodiles are homeostatic for plasma electrolytes and exchangeable Na and water pools in salinities of up to 60‰ (Grigg, 1981; Taplin, in prep., a). The most parsimonious explanation of the observations is that *C. porosus* is not dependent on drinking of either SW or FW to maintain hydromineral balance in SW but must rely on access to a more or less abundant food supply to compensate for evaporative, osmotic and excretory water losses. This conclusion does not, however, preclude the possibility of facultative drinking of FW (for which there is considerable anecdotal evidence) under some circumstances—e.g. crocodiles returning to land after more or less prolonged journeys at sea, where food may not be readily available.

No attempt was made in this study to determine whether or not *C. porosus* drinks brackish water or to measure the range of salinities over which the transition from drinking to not drinking occurs. However, Grigg (1981) noted that marked shifts in cloacal urine composition occurred in crocodiles captured at about 10‰ salinity, the isosmotic point for *C. porosus*. Crocodiles which had access to water of salinity < 10‰ at some stage of the tide (Grigg's SAL1, SAL2 and SAL3 categories) had uniformly high urine NH_4HCO_3 levels and low osmotic pressure, urea, creatinine, uric acid and solids content. Crocodiles from SAL4 (salinity > 10‰ at all times) had low NH_4HCO_3 and high urea, creatinine, uric acid and solids content (Grigg, 1981). It seems very probable on the basis of this evidence that *C. porosus* will drink hypo-osmotic salt water and, in fact, does so in areas where it is exposed to tidal cycling of alternately hypo- and hyper-osmotic water.

The indirect evidence available suggests that many estuarine/marine reptiles avoid drinking SW (see Introduction). There is no reason to expect, however, that this should be a universal phenomenon. Osmoregulation by active ingestion or anal uptake of SW followed by extrarenal excretion of the excess salt is common among marine invertebrates and fish (Spaargaren, 1979; Evans, 1979) and could well occur in reptiles also. Preliminary evidence suggests this might, in fact, be the case for some marine turtles. Both the Loggerhead, *Caretta caretta*, and Green Turtles, *Chelonia mydas*, show much higher rates of Na turnover than other estuarine reptiles when held unfed in SW (Table 2). It has been suggested (Dunson, 1979) that the high rate measured in *Caretta* (Evans, 1973) was due to a severe Na imbalance during the measurement period. However, it is equally possible that marine turtles drink SW and excrete the excess electrolyte through their lachrymal salt glands, as suggested by Holmes and McBean (1964).

Three hatchling *C. caretta* (47–60 g BW) were tested for SW drinking in this laboratory using the same gravimetric technique applied to free-swimming *C. porosus*. When prevented from drinking they showed a net water loss of 122 ± 18 µl/100 g hr compared with a net uptake of 52 ± 9 µl/100 g hr when allowed to drink (unpublished observations). The drinking rate of 174 ± 24 µl/100 g hr in these turtles would contribute 82 µmol/100 g hr to the gross Na influx, a substantial fraction of the measured turnover rate (Table 2). A more detailed study of drinking and osmoregulation in *Caretta* is currently under way in our laboratory and should help establish whether or not this drinking behavior allows it to osmoregulate in SW while unfed.

Despite its obvious importance, the drinking behaviour of aquatic reptiles, particularly estuarine/marine species, has received only superficial attention in previous studies of their osmoregulatory strategies. The evidence that *C. porosus* is able to discriminate SW from FW and, perhaps, hypo- from hyper-osmotic salt water suggests that finely-tuned drinking behavior may play an important role in enabling the species to inhabit the very wide range of salinities (0–70‰ at least) in which it is known to

Table 2. Na turnover rates of unfed, aquatic reptiles in seawater (μmol/100 g/hr). M = marine; E = estuarine; F = freshwater

Species	Usual habitat	Na flux Influx	Efflux	Source
Pelamis platurus	M	8.2	7.1	Dunson (1978)
Caretta caretta	M	—	300	Evans (1973)
Chelonia mydas	M	—	131	Kooistra and Evans (1976)
Nerodia fasciata clarki	E	22.8	—	Dunson (1978)
Cerberus rhynchops	E	11.5	—	*ibid.*
Malaclemys terrapin	E	6–10	—	Robinson and Dunson (1976)
Crocodylus porosus	E	19.0	18.8	Taplin (1982, in prep. b)
Regina septemvittata	F	507	—	Dunson (1978)
Nerodia fasciata pictiventris	F	132	—	*ibid.*
Chrysemys picta	F	8.2	—	*ibid.*

occur (Messel *et al.*, 1981; Taplin, in press, a). Together with the facts that at least one species of marine turtle actively drinks SW and that substantial differences in the partitioning of oral and integumental uptake are evident among FW reptiles, the observations suggest a somewhat greater diversity of drinking strategies among aquatic reptiles than may have been acknowledged previously. Further work on the details of drinking strategies, both within and between taxa, will be useful in cataloguing the range of behaviors represented. However, the study of drinking in isolation from a consideration of overall water and electrolyte budgets is unlikely to add greatly to our understanding of osmoregulation in the group as a whole.

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A TECHNIQUE FOR INVESTIGATING FOOD UTILIZATION IN INDIVIDUAL SMALL AQUATIC INVERTEBRATES

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Abstract—1. A technique is described for measuring in individual small aquatic invertebrates assimilation efficiency and other food utilization characteristics based on the carbon budget.
2. The method, which incorporates a ^{14}C radio-labelling technique, enables continuous monitoring of metabolized (respired) and excreted components to be performed.

INTRODUCTION

This method, which incorporates a ^{14}C radio-labelling technique, is based largely on the work of Kofoed (1975 a,b and personal communication) and was originally designed for the study of assimilation efficiency in *Lymnaea stagnalis* (L.), a freshwater pulmonate snail (Harrison, 1978). It is appropriate for use with any aquatic invertebrate of suitable small size and is especially useful where animals are to be studied individually or in small groups. Food utilization parameters, based on the carbon budget, can be measured at specific intervals throughout any experimental period, without significant interruption.

PRINCIPLES OF THE TECHNIQUE

The experimental animal is fed with material suitably labelled with ^{14}C; respired CO_2 is then continuously collected and all egested and secreted materials are collected at specific periods throughout the experiment. At the end of the experiment the solid materials and the animal itself are digested in nitric acid. Analysis of the ^{14}C content of the various components thus obtained is performed using a scintillation technique. By summing the respired, rejected and incorporated (plus gut content) components the total quantity of material ingested is calculated in terms of radioactive carbon. Using these results assimilation efficiencies may be calculated, and these and other features of the digestive, metabolic and assimilation processes followed with time.

APPARATUS

The essential details of the apparatus are shown in diagrammatic form in Fig. 1. It consists of three closed chambers, constructed from truncated dropping funnels, in a vertical arrangement. The top chamber (a) is the water reservoir and contains a thermometer for monitoring the temperature of the system. This chamber is connected via a glass stopcock to the respiration chamber (b) which contains the animal under study and approximately 40 ml of water. Chambers (a) and (b) are temperature controlled by means of polyethylene tubing coiled tightly around the outside and connected to a pumping water bath with suitably adjusted thermostat. The respiration chamber is connected via a glass stopcock to the acidification chamber (c) which itself leads into a replaceable open collection tube (d). The rubber stopper of the acidification chamber is pierced by a syringe needle through which weak hydrochloric acid may be injected. The respiration and acidification chambers each have an inlet aeration tube and an outlet tube. The reservoir also has an inlet tube for pressure equalization. All three inlet tubes are connected via flow rate control valves to a water vapour-saturated, CO_2-free, filtered air supply. The outlet tubes from chambers (b) and (c) are connected via two-way valves to two pairs of vigreux columns containing a CO_2 absorber suitable for scintillation analysis. Connective piping for the apparatus consists of 1/8 inch glass and polyethylene tubing.

The respiration and acidification chambers both have manoeuverable rinsing tubes (not shown in diagram) inserted through their stoppers. These have angled constricted nozzles through which water may be forced in order to remove any material (e.g. faeces, mucus) which becomes adhered to the inside of the chamber walls.

Seals and joints are made air-tight as necessary by the application of high-vacuum silicone grease.

PROCEDURE

After being fed for a suitable period (e.g. 20 min) with ^{14}C-labelled material, the experimental animal is washed and placed in the respiration chamber. Air is slowly and continuously bubbled through the water in the chamber and collected in a pair of vigreux columns, each containing a small volume (*ca* 10 ml) of CO_2 absorber (ethanolamine in ethylene glycol monomethyl ether, 1:2 v/v). At predetermined intervals the water, containing dissolved and particulate matter, is drained from the respiration chamber (subsequently refilled from the reservoir) into the acidification chamber. Here a small quantity of hydrochloric acid (2 ml of 1N HCl) is added to liberate

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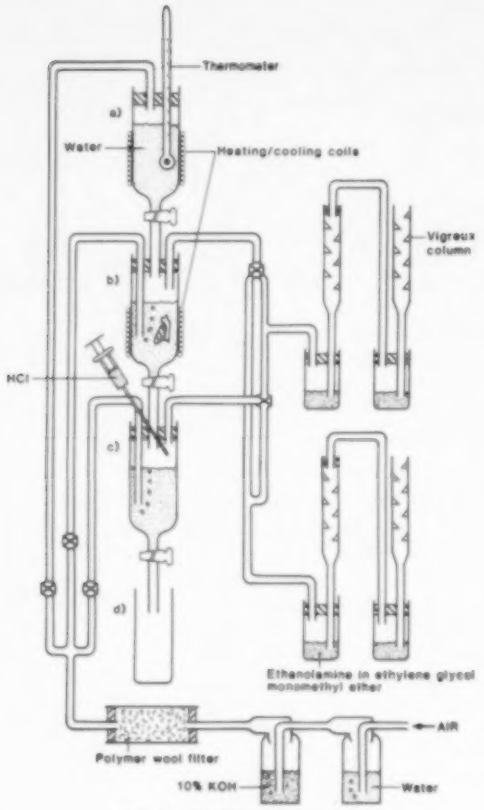

Fig. 1. Diagram of apparatus.

any CO_2 dissolved in the water. This is bubbled off and collected in the same aliquots of absorber, which are then removed and analysed by scintillation counting according to the method of Jeffay and Alvarez (1961). Rejected materials (faeces, urine, mucus, etc.) are collected and centrifuged. Solid materal is digested and analysed according to the rapid nitric acid procedure of Pfeffer *et al.* (1971). Aliquots of liquid supernatant are also analysed for radioactive content. "Quenching" is estimated and corrected for in all cases.

At the end of the experiment the animal is digested using the nitric acid technique. CO_2 released (from a carbonate shell, for example) is also collected and analysed. In this way the total amount of labelled material remaining in the gut or assimilated into the tissues is determined. Total ingested material is calculated as the sum of the total amounts respired, rejected and incorporated in the animal, plus final gut contents. Actual amounts of carbon digested and metabolized are assumed to be proportional to the quantities of ^{14}C detected, presented in terms of decompositions per min. A diagrammatic representation of the experimental procedure is shown in Fig. 2.

Cumulative proportions of ^{14}C respired and contained in rejecta may be calculated for specific time intervals and assimilation efficiencies estimated, for example, according to the formula of Petrusewicz and Macfadyen (1970):

$$A = (C - (F + U + M))/C$$

where A = assimilation efficiency, C = total consumption, F = true faeces, U = urine (nitrogenous excretion) and M = other metabolized products such as mucus. ($F + U + M$ = "rejecta"). Further interesting ratios which can be studied using this method are the proportion of ^{14}C ingested which is respired, and the ratio of amount respired to amount apparently available for metabolism in any particular time period.

FURTHER COMMENTS

The total amount of radioactive carbon ingested by the experimental animal is dependent upon the food material used and the amount of ^{14}C incorporated in it, the rate of consumption by the animal and the duration of the feeding period. It is important to

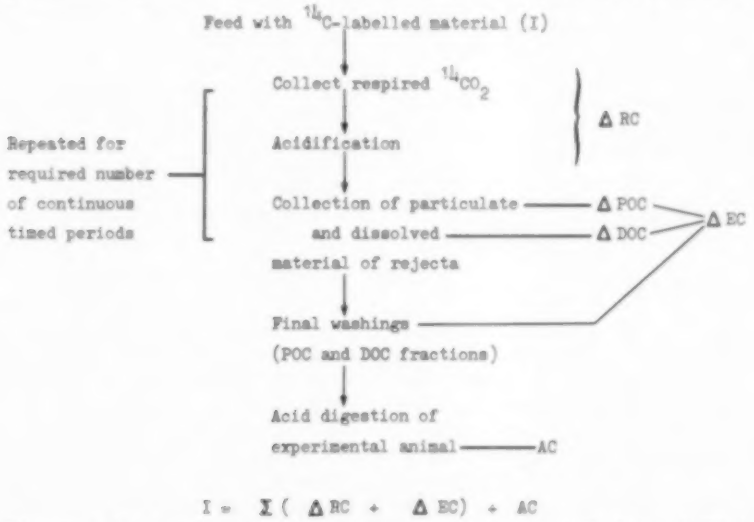
Feed with ^{14}C-labelled material (I)
Collect respired $^{14}CO_2$
Δ RC
Repeated for required number of continuous timed periods
Acidification
Collection of particulate and dissolved material of rejecta
Δ POC
Δ DOC
Δ EC
Final washings (POC and DOC fractions)
Acid digestion of experimental animal
AC
$I = \Sigma(\Delta RC + \Delta EC) + AC$

Fig. 2. Diagrammatic representation of experimental procedure. Legend: I = total ^{14}C ingested; ΔRC = respired ^{14}C fraction; ΔPOC = particulate organic carbon fraction; ΔDOC = dissolved organic carbon fraction; ΔEC = total ^{14}C fraction of rejecta ($\Delta POC + \Delta DOC$); and AC = incorporated ^{14}C + remaining gut contents.

optimize this quantity to ensure adequate "counts" in each component studied; this must be done by trial and error in preliminary experiments.

A major requirement of the technique is that all excess labelled food is removed from the animal before the experiment proper is begun. Failure to do so may result in significant experimental error, reflected by very high ^{14}C counts in the rejecta of the initial periods. It is also important to prevent the experimental animal from ingesting its own faeces since re-ingestion of labelled defaecated material would cause a slight increase in assimilation efficiency; the time-scale of faeces and metabolite production would also be affected. A suitable gauze placed in the bottom of the respiration chamber helps prevent this occurrence.

Small errors may be incurred by the assumption that labelled material is recovered from the acid digests with 100% efficiency. Pfeffer *et al.* (1971) quote a value of $99.3 \pm 2.9\%$ for efficiency of recovery of ^{14}C from the standard digest, but significant reductions in this figure may occur through the experiment, causing a final small increase in the estimated values of assimilation efficiency. Errors associated with the design of the apparatus are considered to be slight: potential "dead-space" in the apparatus may, through diffusion effects, delay the collection of $^{14}CO_2$ respired in any particular period, but this is probably insignificant.

A practical application of this method is described in full by Harrison (1978). It is suggested that the technique is especially valuable in comparative studies.

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ENERGETICS AND OSMOREGULATION IN THE CATFISH, *ICTALURUS NEBULOSUS* AND *I. PUNCTATUS*

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Abstract—1. Groups of brown bullhead catfish (*Ictalurus nebulosus*) and channel catfish (*I. punctatus*) were acclimated to different concentrations (6, 120, 200 mOsm/kg) of either diluted artificial seawater (DASW) or $MgSO_4$ solutions in order to derive the metabolic costs of osmotic and ionic regulation in fresh water.

2. Oxygen consumption declined when the osmotic and/or ionic gradient between the external medium and internal body fluids decreased. In the DASW solutions the reductions were 16.8% and 28.6% in the 120 and 200 mOsm/kg solutions, whereas in $MgSO_4$ solutions the reductions were 13.2% and 27.1%, respectively, indicating that the cost of living in a dilute medium is largely that of osmotic regulation.

3. Both urine flow rate and glomerular filtration rate (GFR) decreased greatly (83% and 93% in the 200 mOsm/kg DASW amd $MgSO_4$ solutions, respectively) as the medium concentration decreased.

4. We propose that the major portion of the measured decreases in oxygen consumption is a result of the reduction in the active reabsorption of Na^+ and K^+ by the kidney due to the reduced GFR.

INTRODUCTION

Freshwater fish have blood concentrations of close to 300 mOsm/kg but live in a medium of less than 10 mOsm/kg. This situation has a two-fold consequence: efflux of essential ions and influx of water. In order to compensate for this ion loss and water gain, metabolic energy must be expanded. It follows that animals living in media that cause a decrease in these fluxes should expend less energy. Madan Mohan Rao (1968), using rainbow trout, *Salmo gairdneri*, found a decrease of 20% in the oxygen consumption of fish in isomotic diluted seawater as compared to those in fresh water. Farmer and Beamish (1969) obtained similar results with *Tilapia nilotica*. Maceina *et al.* (1980), using grass carp, *Cytenopharyngodon idella*, and Cordier and Maurice (1957), using tench, *Tinca vulgaris*, both of which are relatively stenohaline freshwater fishes, found even greater decreases of 31% and 27%, respectively.

Decreased metabolic rate has also been observed in two species of invertebrates in response to similar experimental conditions. Schwabe (1933) measured a 40% decrease in oxygen consumption in the crayfish, *Astacus fluviatilus*, upon transfer from fresh water to isomotic saline. Under similar conditions Flemister and Flemister (1951) found a 30% decrease in the oxygen consumption of the crab, *Ocypode albicans*.

These studies, however, have not addressed the problem, recognized by Fry (1976), of distinguishing the cost of ion transfer from that of water transfer. In the case of a teleost fish placed in isosmotic dilute seawater both the osmotic and ionic (mainly Na^+ and Cl^-) gradients between the fish and the external medium have been reduced to a minimal level, whereas in a medium of similarly high osmolarity, but containing minimal amounts of Na and Cl, there would be an equivalent decrease in the osmotic gradient without a change in the ionic gradient. Thus, the difference between the metabolic rate of a fish in fresh water and the metabolic rate of a fish in an isosmotic medium without NaCl would be the metabolic cost of osmotic regulation alone. This value for water regulation, subtracted from the value for total regulation (ionic and water), would theoretically yield a value for the fraction of metabolic energy required for ionic regulation.

With the previous analysis in mind, we undertook in this study to determine the metabolic rate of brown bullhead catfish (*Ictalurus nebulosus*) in media containing elevated concentrations of NaCl or $MgSO_4$ so that these metabolic rates could be compared with freshwater values. Concurrent measurement of Na^+ efflux, urine flow and composition and glomerular filtration rate were made so that the mechanism by which reductions in metabolic rate were effected could be determined. Channel catfish (*I. punctatus*) were used for the latter measurements as well.

The brown bullhead and channel catfish are considered typical stenohaline freshwater fish; nevertheless, they are commonly found in brackishwater lakes and estuaries (Norton and Davis, 1977). In addition, these hardy and stress resistant (Walker, 1971; Norton and Davis, 1977) species are tolerant of experimental manipulation.

MATERIALS AND METHODS

Brown bullhead catfish, *Ictalurus nebulosus*, (30–190 g) were obtained from local hatcheries. They were held in aquaria of 80 liter capacity in which water was continuously recirculated through filter floss and activated carbon. Water

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Table 1. Sodium, chloride and potassium concentrations in the experimental media

Medium		Concentration (meq/l) Sodium	Chloride	Potassium
Fresh water 6 mOsm/kg		0.5	5	0.4
120 mOsm/kg	DASW	55	60	2.3
	$MgSO_4$	1.5	5	1.5
200 mOsm/kg	DASW	93	100	4.3
	$MgSO_4$	0.2	6	3

temperature was maintained at 21°C±0.5°C. A 12:12 light:dark photoperiod was maintained throughout the experimental period. Normal *ad libitum* feeding with trout chow was suspended at least 48 hr before an experiment. In some experiments (GFR and Na efflux) similar size channel catfish *I. punctatus* were also used due to unavailability of bullheads. Results from the two species were combined, based on their physiological similarity noted in this study and in the literature (Norton and Davis, 1977).

The fresh water used was dechlorinated ("aged") Bloomington tap water. Dilute artificial seawater (DASW) solutions were made up by adding appropriate amounts of artificial sea salt (Instant Ocean) to tap water. The $MgSO_4$ solutions were made up in the same way. Total osmotic concentration was maintained to within ±5 mOsm/kg. Typical Na, Cl and K concentrations are presented in Table 1.

Respirometers consisted of 3- to 4-liter, sealable glass or Plexiglass chambers. Aerated, temperature-controlled "home-tank" medium from a 4-liter reservoir was circulated through the chambers by a variable speed pump (FMI) and then led back to the reservoir. Flow rate was based on body weight (1 ml/min per gram) in order to maintain a conveniently measureable difference between inlet and outlet water oxygen content.

Experimental protocols

Oxygen consumption. The following procedure for determining oxygen consumption was performed on fish that had been in laboratory aquaria (fresh water) for at least 2 weeks after receipt from a supplier.

Each fish of a group of three was placed in a separate, darkened respirometer in the early morning. Oxygen consumption was measured in the early afternoon, a time of day when *I. nebulosus* has been shown to be least active (Crawshaw, 1975; Eriksson and van Veen, 1980). Afterwards, fish were returned to their aquaria. This procedure was repeated on three consecutive days. On the third day, after the oxygen consumption measurement, all three fish were weighed, and a blood sample was taken from one. After the fish had been fed, the concentration of the aquarium water was increased to the next level by the addition of the appropriate salt over a period of 2 days. Three days were then allowed for acclimation (longer acclimation periods, up to one week, yielded similar results). Using the same fish, this procedure was repeated at the other two acclimation concentrations, but each time the blood sample was drawn from a different fish of the three.

This protocol was used with three groups for a total of nine fish in each medium.

The partial pressured of oxygen in each medium was measured with a polarographic O_2 electrode (Radiometer). This instrument was calibrated daily. Water samples were analyzed at 10-min intervals until two similar (±1 mm Hg) consecutive readings were obtained. The Winkler method was used to determine oxygen solubility in the various media.

The rate of oxygen consumption was determined by the following equation:

$$\dot{M}O_2 = V(C_i - C_e)/W$$

where $\dot{M}_{O_2}$ = oxygen consumption, mg O_2/(g × hr); V = flow rate, l/hr; C_i = oxygen concentration of water entering chamber, mg O_2/l; C_e = oxygen concentration of water leaving chamber, mg O_2/l; W = weight of the fish, g.

Urine flow. A catheter of PE 60 intramedic tubing (Clay–Adams) was inserted into the urinary bladder of an anesthetized fish through the urinary or urogenital papilla (depending on sex and species) and then fixed in place by sutures to the anal fin. The end of the catheter was heat flared to prevent tissue damage and leakage.

The fish was then placed in a Plexiglass chamber through which aerated, filtered, "home tank" medium was recirculated. The catheter was run through a silicone sealed opening at one end leading in to a 1- or 10-ml pipette. Sampling was not begun for 4–6 hr post-insertion to allow for the dimunition of handling-induced diuresis. Samples were taken after flow rate had remained constant for 2–3 hr and were then frozen for subsequent ion analysis.

GFR. ^{14}C-labelled polyethylene glycol (PEG) (New England Nuclear), was used as a marker to determine glomerular filtration rate. Following catheterization, 1.0 μCi of the ^{14}C-labelled PEG in 50 μl of isotonic saline was injected into the peritoneal cavity. After allowing 10–16 hr for distribution throughout the animal (Walker, 1971), urine and plasma samples were taken. Aliquots (0.2 ml) of each were suspended in scintillation fluid (Insta-Gel, Packard Instruments) for beta counting. *GFR* was calculated from the following equation:

$$GFR = v \times (u/p)$$

where GFR = ml/min; v = rate of urine production, l/min; u = cpm/ml urine; P = cpm/ml plasma.

Na^+ *efflux.* This was calculated as follows (Eddy *et al.*, 1980):

$$K_{Na} = (1/T) \times \ln((C_e - C_T)/C_e)$$

where K_{Na} = total Na^+ flux; T = time; C_e = counts in medium at isotopic equilibrium with body fluids; C_T = counts in medium at time T.

Blood. Blood samples (0.5–1.0 ml) were drawn into sodium heparinized syringes from the caudal artery of an anesthetized fish. Plasma was obtained by centrifugation and then immediately frozen.

Anesthesia. Anesthesia was accomplished through the injection of a barbiturate, methohexital sodium (Brevital Sodium, Eli Lilly & Co.), dissolved in distilled water to a concentration of 10 g/l. Dose was weight dependent, 0.1 ml/100 g. Recovery occurred within one-half hour.

Ions and osmolarity. Na^+ and K^+ analyses were performed with a flame photometer (IL 143), chloride was determined by titration (Buchler-Cotlove Auto Titrator) and osmolarity was measured with either a freezing-point (Advanced Instrument Inc.) or a vapor pressure (Wescor) osmometer.

Radioactivity. ^{22}Na was measured with a gamma scintillation counter (Beckman Gamma 4000). ^{14}C-labelled PEG was measured in a liquid scintillation counter (Beckman LS-233). The LS-233 automatically compensates for quenching through the use of an external standard.

RESULTS

Oxygen consumption

The oxygen consumption data, obtained from 18 fish, nine in each medium, are presented in Fig. 1. Large decreases in oxygen consumption were observed as the osmolarity of each medium was increased. The decreases measured in the dilute artificial seawater (DASW) solutions are not significantly greater than those measured in the $MgSO_4$ solutions.

Plasma ions and total osmotic concentration

Na^+, Cl^-, ($[Na^+]p$, $[Cl^-]p$) and total osmotic

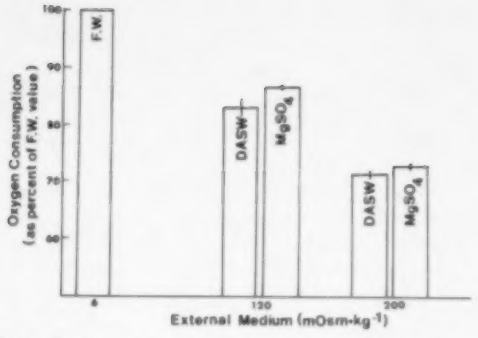

Fig. 1. Oxygen consumption (as a percent of the fresh water value) in relation to the external medium osmolarity. The difference in the decrease in oxygen consumption between the two media is significant only in the 120 mOsm/kg solutions. Vertical lines represent standard error of the mean.

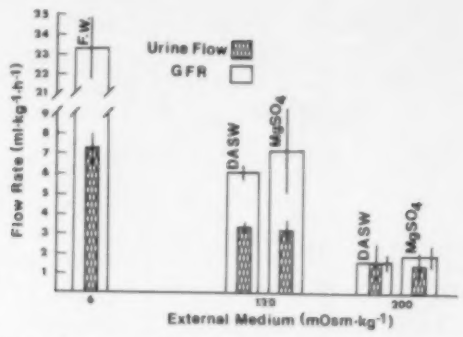

Fig. 2. Urine flow and glomerular filtration rates in relation to external medium osmolarity. There is no significant difference between the values for the DASW and $MgSO_4$ solutions. Vertical lines represent standard error of the mean.

concentration in the plasma rose as the ambient DASW concentration increased (Table 2). These increases were significantly different from fresh water ($P < 0.05$; Mann–Whitney U-test) only at the higher concentration. In the $MgSO_4$ solutions, plasma ion concentrations remained at freshwater levels whereas the elevation in total osmotic concentration was significant ($P < 0.05$; Mann–Whitney U-test). The total osmotic concentration, however, did increase; the difference between the sum of $[Na^+]p$, $[Cl^-]p$ and $[K^+]p$ and the total osmotic concentration was 10 mOsm and 30 mOsm in the 120 and 200 mOsm/kg $MgSO_4$ solutions, respectively. We assume that this difference between the sum of the ion concentrations and the total osmotic concentration is at least partly attributable to Mg^{2+} and SO_4^{2-} ions.

Urine flow and glomerular filtration rate

Urine flow rate decreased significantly ($P < 0.01$; Mann–Whitney U-test) as total osmotic concentration increased in both media (Fig. 2). The difference between the flow rates in each medium was not significant. The average percentage decrease in urine flow rate for each medium from fresh water to 120 mOsm/kg and from 120 to 200 mOsm/kg was similar, 55% and 53%, respectively. The average decrease in flow rate from fresh water to 200 mOsm/kg was 79.5%.

The glomerular filtration rate exhibited a large decrease as the total osmotic concentration increased in each medium (Fig. 2). As with the urine flow rate, the percentage decreases from fresh water to 120 mOsm/kg and from 120 to 200 mOsm/kg were similar, 72% and 73%, respectively. The average decrease of GFR from fresh water to 200 mOsm/kg was 93%.

Urine Na^+, Cl^-, K^+ ($[Na^+]_u$, $[Cl^-]_u$, $[K^+]_u$) and total osmotic concentrations were very low in freshwater (Table 3). In the DASW solutions the total osmotic concentration of the urine (sum of $[Na^+]_u$, $[Cl^-]_u$ and $[K^+]_u$) is equivalent to the ambient osmotic concentration. Although the total osmotic concentration of the urine in the $MgSO_4$ solutions

Table 2. Sodium, chloride, potassium and total osmotic concentrations of the plasma of fish acclimated to the various experimental media. Values are means ±1 SE. Sample size is 6 for the fresh water values and 3 for the others

		Sodium (meq/l)	Chloride (meq/l)	Potassium (meq/l)	Osmolality (mOsm/kg)
Fresh water		134±2	117±3	3.7±0.5	260± 5
120 mOsm/kg	DASW	139±2	123±4	3.1±1.1	270± 2
	$MgSO_4$	135±3	114±2	2.8±0.1	271±0.4
200 mOsm/kg	DASW	142±1	130±6	3.6±0.8	283±11
	$MgSO_4$	134±3	114±2	3.2±0.1	281± 2

Table 3. Sodium, chloride, potassium and total osmotic concentrations of the urine of fish acclimated to the various experimental media. Values are means ±1 SE. Sample size is 9 for the fresh water values and 3 for the others

		Sodium (meq/l)	Chloride (meq/l)	Potassium (meq/l)	Osmolality (mOsm/kg)
Fresh water		5.5±0.6	3.8±0.6	0.31±0.04	10.8±0.6
120 mOsm/kg	DASW	60 ±1	52 ±2	6.6 ±1	119 ±3
	$MgSO_4$	6.0±0.9	3.5±0.5	0.33±0.09	100 ±1.8
200 mOsm/kg	DASW	100 ±3	90 ±1	6.4 ±0.6	197 ±3
	$MgSO_4$	8.5±1.3	4.0±7	0.7 ±2	200 ±5

Table 4. Rate of reabsorption of electrolytes by the kidney in each medium

		Rate of reabsorption (meq/(kg × hr))			
		Na	Cl	K	Total
Fresh water		3.080	2.70	0.084	5.864
120 mOsm/kg	DASW	0.640	0.567	0	1.207
	$MgSO_4$	0.946	0.804	0.019	1.769
200 mOsm/kg	DASW	0.07	0.066	0	0.136
	$MgSO_4$	0.247	0.214	0.005	0.466

behaved similarly in response to increasing ambient osmolarity. $[Na^+]_u$, $[Cl^-]_u$ and $[K^+]_u$ did not increase in the 120 mOsm/kg solution and only slightly in the 200 mOsm/kg solution. We assume that Mg^{2+} and SO_4^{2-} ions were the contributing factors to the high total osmotic concentration of the urine.

The quantity of electrolyte reabsorbed was determined by subtracting the quantity of electrolyte present in the urine (urine flow × concentration) from the quantity present in the glomerular filtrate (GFR × plasma concentration). Table 4 presents such values for Na^+, Cl^- and K^+ in the various media.

Na *Efflux*

The data presented in Fig. 3 represent total Na efflux, i.e. via gills and kidneys. Efflux in the urine, however, accounts for 10% of the total in fresh water, and even less in the other media. In the DASW solutions there was a substantial increase in efflux, whereas in the $MgSO_4$ solutions efflux remained at freshwater levels.

DISCUSSION

Osmotic gradient

Although catfish can tolerate and apparently thrive in DASW solutions at concentrations of 260 mOsm/kg and higher they begin to exhibit adverse reactions (e.g. increased mucus production, mottling and death) in concentrations of $MgSO_4$ higher than 220–230 mOsm/kg. It is for this reason that we decided to use 200 mOsm/kg as the maximum concentration for both media. The catfish exhibited no adverse reactions in either medium at this concentration. The gradient between external medium and internal body fluids at an external concentration of 200 mOsm/kg is equivalent to the gradient existing at higher external concentrations. In DASW solutions above 200 mOsm/kg catfish maintain a gradient of 66 mOsm/kg between the external medium and internal body fluids (e.g. in DASW of 260 mOsm/kg plasma concentration was found to be approximately 320 mOsm/kg, unpublished data). Under similar conditions goldfish maintain their plasma osmolarity 20 mOsm/kg above the medium (Lahlou *et al.*, 1969) and grass carp maintain their plasma osmolarity 50 mOsm/kg above the medium (Maceina *et al.*, 1980). Maintaining such a gradient between external medium and internal body fluids assures a constant osmotic inflow of water.

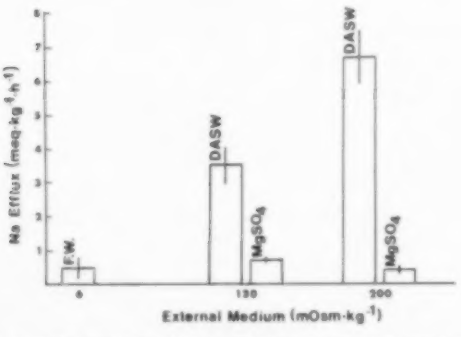

Fig. 3. Total sodium efflux in relation to external medium osmolarity. Vertical lines represent standard errors of the mean.

Kidney function

The low concentration of electrolytes in the urine of freshwater-adapted fish (Table 3) is expected because of the need for electrolyte conservation in this dilute medium. As the concentration of the DASW solutions increases, the need for conservation of these electrolytes decreases, resulting in an increase in their concentrations in the urine. In the $MgSO_4$ solutions the need for conservation is maintained, as evidenced by the low concentrations of the electrolytes in the urine. Unfortunately, the means for measuring Mg^{2+} and SO_4^{2-} concentrations were not available; however, due to the low concentrations of Na^+, Cl^- and K^+ and the high total osmotic concentration in the urine, we assume that $MgSO_4$ is present in the urine in high concentrations. If so, this would indicate (in conjunction with low concentration of $MgSO_4$ in the plasma), that these divalent ions are being actively secreted by the kidney tubules. This finding is in agreement with work done by Hickman (1968) and Rankin *et al.* (1980) who also found evidence for the Mg^{2+} and SO_4^{2-} secreting ability of the fish kidney.

The decrease of GFR as osmotic concentration increased in both media has the important effect of decreasing the work load of the kidney by the concomitant reduction in the entrance of fluid and electrolytes (Na^+, Cl^- and K^+). In catfish acclimated to freshwater, an electrolyte load of 5.86 meq/(kg × hr) is introduced into the kidney. This value is based on a GFR of 23.3 ml/(kg × hr) and a filtrate concentration of 252 meq/l, equal to the combined concentrations of Na^+, Cl^- and K^+ in the plasma. Similarly, Walker (1971) measured a GFR of 22.8 ml/(kg × hr) and a similar filtrate concentration for the brown bullhead catfish.

The large decrease in GFR in response to increasing osmotic concentration in the 200 mOsm/kg concentrations of each medium reduces greatly the large quantities of water and electrolytes the kidney must process. This reduction manifests itself as a reduction in urine production and electrolyte reabsorption. Urine production dropped by 45% in the 120 mOsm/kg solutions and by 80% in the 200 mOsm/kg solutions. The latter figure is similar to a decrease of 78% noted by Lahlou *et al.* (1969) in goldfish, *Carrasius auratus*, acclimated to a hyperosmotic dilute seawater.

Although the total quantity of electrolytes entering the kidney at any one osmotic concentration is similar (due to similar GFR's), the amount of monovalent ions reabsorbed is greater for fish in the $MgSO_4$ solutions. In effect, simply viewed and for purposes of comparison, the only difference in the response of the kidney to increasing concentrations of either medium

is a lower rate of reabsorption of electrolytes in the DASW solutions. The significance of this difference in relation to the metabolic rate will be discussed later in this section.

Na^+ *efflux*

The Na^+ efflux rate from freshwater-acclimated catfish (0.43 meq/(kg × hr), Fig. 3) is similar to that of other freshwater-adapted fish (e.g. 0.27 for the goldfish (Lahlou *et al.*, 1969) and 0.36 for the African catfish, *Clarius mossambicus* (Eddy *et al.*, 1980). $MgSO_4$ appears to have no effect on Na^+ efflux (Fig. 3), because the efflux rate in these solutions remains at freshwater levels. In fish acclimated to the 120 and 200 mOsm/kg DASW solutions, the Na^+ efflux (Fig. 3) increases to 3.48 and 6.65 meq/(kg × hr), respectively. Comparable increases have been observed in other stenohaline fishes, e.g. goldfish (Lahlou *et al.*, 1969) and in euryhaline fishes, e.g. the flounder, *Platichthys flesus*, (Potts and Eddy, 1973). These large increases in the efflux of Na have been attributed to an "exchange diffusion" effect, notably by Motais *et al.* (1966). As this process appears to require little or no metabolic energy (Ussing, 1960), it does not introduce any difficulties into the interpretation of our results.

Oxygen consumption

For the purposes of this discussion, the metabolic rate of a fish in fresh water can be partitioned between the metabolic costs of ionic regulation, the cost of water regulation, and "other costs" that should be more or less independent of changes in the osmotic or ionic concentration of the medium. By placing the fish in a dilute seawater solution at 200 mOsm/kg containing about 97 mM NaCl, the osmotic and ionic difference between the fish and its environment was reduced on the order of 65%. Reductions in ionic and osmotic gradients resulted in a 29% reduction in metabolic rate. Extrapolating to a complete reduction in gradient yields a 45% reduction in metabolic rate. Although these figures are approximate, they clearly show that of a freshwater fish's total standard metabolism, on the order of 50% is devoted to the tasks of regulating internal salt and water content.

When a fish is in a medium containing $MgSO_4$, the osmotic burden on the fish is reduced although the ionic gradient that the fish must deal with remains unchanged. Thus, by subtracting the MR of fish in $MgSO_4$ (reduced osmotic burden) form the MR of fish in DASW (reduced osmotic and ionic burdens), one obtains an indication of the relative cost to the fish of non-water related ionic regulation. Our results (Fig. 1) show that at external concentrations of 160 and 200 mOsm/kg, there is no significant difference between the MR of fish in DASW and $MgSO_4$. We therefore conclude from these results that the metabolic cost of ionic regulation not related to water regulation is quite small in comparison to the total cost of osmotic regulation in freshwater fish.

For a fish living in fresh water, the gills must replace salts that are lost by diffusion across the body surface as well as salts that are lost in the urine. The sum of these two losses is the value of efflux measured in this study. For both the fresh water fish and the fish in $MgSO_4$ solutions, rates of Na efflux were of the order of 0.5 meq/(kg × hr), with less than 10% of that value being lost in the urine. Thus, the major site of sodium loss by these catfish was across the gills and other areas of the body surface. Compared to these small sodium losses that the gills must make up, the kidney tubules (and perhaps the bladder) of freshwater catfish can be shown to be transporting 3.1 meq Na/(kg × hr). This finding that the rate of sodium transport by the kidneys far exceeds the rate of sodium transport by the gills is consistent with the previously mentioned measurements showing that the metabolic cost of living in a freshwater environment (both osmotic and ionic problems) exceeds the costs of dealing with the exclusively ionic problems encountered in $MgSO_4$.

Thus, with regard to the costs of osmotic and ionic regulation, we see that the major cost to a freshwater catfish is associated with "descalinating" its kidney filtrate (i.e. producing a dilute urine), and that the cost of replacing the sodium lost in the urine is quite small by comparison. Also, our study clearly shows that although the distinction between osmotic and ionic regulation is routinely made, the burden of osmotic regulation is, in the final analysis, largely ionic in nature. Indeed, to a certain extent, ionic regulation can be thought of as a problem of compensating for external salt losses whereas osmotic regulation is largely a problem of dealing with internal *salt* transport despite the appelative emphasis on *water*.

To conclude, the major fraction of the metabolic cost of osmoregulation for the freshwater-adapted catfish can be attributed to the energy used by the kidney to produce a dilute urine. A much smaller fraction (< 5%) of total metabolic energy is expended by the gills to replace the small quantities of ions lost in the urine and through the gills.

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COMPARATIVE TEMPERATURE AND WATER RELATIONS OF *TENEBRIO OBSCURUS* LARVAE (COLEOPTERA: TENEBRIONIDAE)

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Abstract—1. Experiments were conducted in order to determine the upper and lower lethal temperatures, and the effects of temperature and relative humidity on the survival capacity and cuticular permeability of *Tenebrio obscurus* larvae.

2. RH has a greater effect on survival capacity at high temperature extremes than at low temperature extremes. At 12% RH, the upper and lower lethal temperatures were 39.2 and −4.4°C, respectively. At 72% RH, they were 42.6 and −5.9°C, respectively.

3. At cold temperatures, relatively moist conditions (72% RH) enhance survival. Temperature extremes are more limiting for survival under xeric conditions in the larvae of this species. The combination of high temperatures and 72% RH has a greater effect on the survival capacity of this species than does the combination of cold temperatures and 72% RH.

4. Under dry air conditions, these larvae exhibit cuticular permeabilities that fall within the range reported for xeric-adapted arachnids and insects and some mesic spiders. Water loss (weight loss) within the first 12 hr of desiccation was greater at higher humidities. Rates of water loss decrease following initial loss.

5. Fossoriality is an effective mechanism for decreasing rates of water loss in these larvae.

INTRODUCTION

Although there are several reviews concerning temperature compensation and water relations in ectotherms (Cloudsley-Thompson, 1970; Edney, 1977; Weiser, 1973), many invertebrate groups have not been extensively studied. Previous studies have shown that the distribution and activity of terrestrial arthropods is strongly influenced by the capacity of an arthropod to compensate for evaporative water loss (Edney, 1977; Punzo and Jellies, 1983). Several recent studies have confirmed the relationships between environmental conditions in which arthropods are found and their physiological adaptations (Cohen, 1982a; Hadley *et al.*, 1981; Jungreis *et al.*, 1982; Punzo and Mutchmor, 1980; Warburg *et al.*, 1980; Wilson *et al.*, 1982). Additional studies have shown that cuticular permeability represents an evolutionary parameter closely related to environment stress, especially in xeric-adapted arthropods (Cohen *et al.*, 1981; Hadley *et al.*, 1981; Punzo and Jellies, 1983).

The species investigated in the present study, *Tenebrio obscurus* (Fabricius), a beetle frequently found as a pest of stored grain (Dillon and Dillon, 1961), is often exposed to localized microhabitat conditions very similar to those normally associated with desert conditions. Many terrestrial arthropods resist the desiccating properties of the air by remaining in contact with moist substrates, fossoriality, or by the possession of cuticular lipids that effectively decrease evaporative water loss. One aspect of temperature regulation that is directly relevant to water balance is evaporative cooling, which is most effective as a short-term compensation and can be very effective for counteracting high, stressful temperatures. The capacity for evaporative cooling is a function of cuticular permeability. Generally, larger arthropods can utilize evaporative cooling more effectively than those of smaller size (Punzo and Mutchmor, 1978). The problem of cuticular permeability is further intensified by small body size such as that exhibited by ticks, mites and small insects (Edney, 1977) as well as spiderlings (Punzo and Jellies, 1983). To compensate for this, some terrestrial arthropods have evolved mechanisms for absorbing water vapor (Machin *et al.*, 1982).

The present study concerns the temperature and water relations of the darkling beetle, *T. obscurus*, a closely related species to the more widely known mealworm beetle, *Tenebrio molitor* (Linné). Both of these coleopterans can be serious pests of stored grain, and both are commonly found throughout North America (Dillon and Dillon, 1961; Howard, 1955). The temperature and water relations of *T. molitor* have previously been investigated (Punzo and Mutchmor, 1978, 1980). However, with respect to *T. obscurus*, little information is available with respect to its thermal biology. Because of the close taxonomic relationship between these two species as well as their sympatric distributions and their simultaneous occurrence in granaries, storehouses and flour meal, an investigation of the temperature and water relations of *T. obscurus* larvae (the most destructive life-cycle stage) was undertaken.

MATERIALS AND METHODS

The insects used in this study were taken from laboratory rearing colonies maintained at 22°C. Insects were reared in plastic containers as described by Punzo and Mutchmor

(1980). Temperature and relative humidity (RH) analyses were conducted on the larger (older) larval stages (14–32 mm) of *Tenebrio obscurus*. Temperatures were obtained by using a Precision Model 816 environmental chamber (Chicago, IL). All tests were conducted under conditions of constant darkness. RH within the chamber was controlled through the use of saturated salt solutions (Winston and Bates, 1960): LiCl (12%), and NaCl + KCl (72%). Salt solutions were placed in plastic trays in the chamber and RH checked periodically with hydrion paper.

Lethal temperature and survival capacity determinations

Upper (ULT_{50}) and lower (LLT_{50}) lethal temperature determinations were obtained for *T. obscurus* larvae according to the method described by Schmidt-Nielsen (1964). Five larvae were placed in each of 4 plastic dishes provided with meal. Two replicates (20 larvae/replicate) were conducted on a total of 40 larvae at RH values of 12 and 72%. For LLT_{50} determinations, 15 different groups of 40 larvae were exposed to low temperatures ranging from −1.0 to −8.0°C at 0.5°C intervals (40 larvae at each combination of temperature and RH), for a period of 1 hr. Following the period of exposure, the larvae were removed from the chamber, isolated and returned to normal rearing conditions. The number surviving after a 24 hr period was recorded as described by Fritz and Punzo (1976). Identical procedures were employed for ULT_{50} determinations. Larvae were exposed to high temperatures ranging from 36 to 45°C at 1.0°C intervals, at 12 or 72% RH.

The above data on the combined effects of temperature and RH allowed for the determination of lethal temperatures as well as for an analysis of the effects of these parameters on survival capacity (Figs 1 and 2).

Evaporative water loss/cuticular permeability

Fifteen larvae were placed individually in plastic cylinders (one group of 15 larvae for each experimental condition) provided with polyester fiber plugs at one end and silicone plugs at the other end. Each cylinder was provided with small holes for air circulation. Individual larvae were weighed on a Sartorius balance to the nearest 0.005 mg and re-weighed at 12-hr intervals for 48 hr. Water loss and weight loss were regarded as equivalent (Punzo and Jellies, 1983). Larvae were prevented from feeding for 24 hr prior to testing and defecation was minimal or absent.

Desiccation experiments (Tables 2 and 3) were conducted in a constant temperature chamber under constant darkness at various humidities. Silica gel was used to obtain extremely dry (2% RH) conditions. Saturated salt solutions (Winston and Bates, 1960) were used to obtain the following humidities: NaI (33%), NaCl + KCl (72%), and $K_2Cr_2O_7$ (98%). The apparatus and procedures used for desiccation experiments have been described in a previous paper (Punzo and Jellies, 1983). The surface area of individual larvae was obtained using the relationship, $S = kW^{2/3}$, where S represents surface area (cm^2), W the weight (mg), and k a species-specific constant (Edney, 1977). To obtain a value for k, larvae of known weight were cleared in KOH. Their exoskeletons were spread over paper provided with 0.25 mm squares as described by Punzo and Jellies (1983) and measured with a dissecting microscope. The k value obtained for the larvae of this species was 0.201 $cm^2/mg \pm 0.02$ SD.

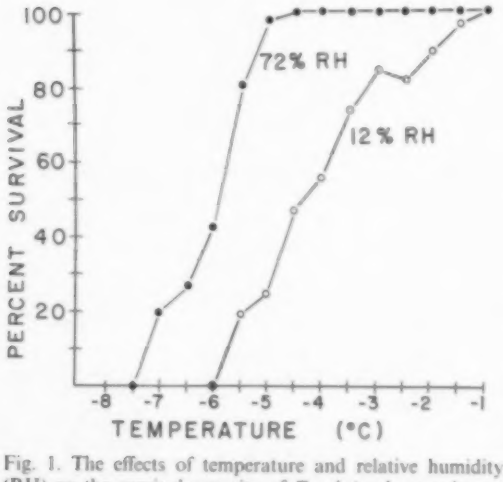

Fig. 1. The effects of temperature and relative humidity (RH) on the survival capacity of *Tenebrio obscurus* larvae under conditions of low temperature stress.

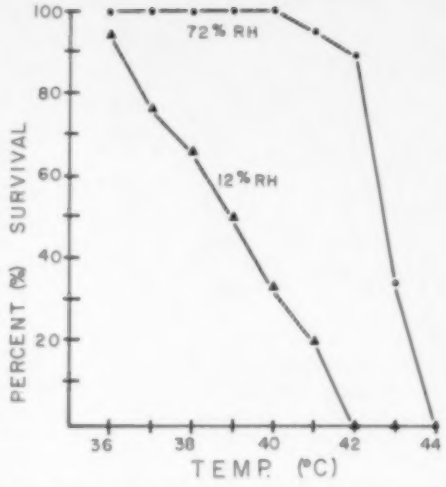

Fig. 2. The effects of temperature and relative humidity (RH) on the survival capacity of *Tenebrio obscurus* larvae under conditions of high temperature stress.

To evaluate the effect of RH on cuticular permeability, partially desiccated larvae from the 33% RH group were exposed to 98% RH conditions and their weights were recorded at 12-hr intervals for 48 hr. The larvae were then returned to the 33% RH condition and weighed after 24 hr. Temperature was kept constant (25°C) during these experiments since temperature has been shown to alter the structure of arthropod cuticle (Hadley, 1980). Saturation deficit was calculated according to the method reported by Hill (1976).

Another series of experiments were conducted in order to assess the effectiveness of burrowing as a possible mechanism to resist evaporative water loss (Fig. 3). Two groups (each group, $N = 10$) of larvae (14 mm length) were tested individually for weight loss ($mg\,g^{-1}\,hr^{-1}$) under stressful conditions (38°C, 12% RH). The larvae of 1 group were tested in plastic containers without grain and thus prevented from burrowing (Fig. 3, unburrowed). In the other group, plastic containers were provided with grain to a depth of 6 cm in order to allow the larvae to burrow in response to high temperature and low moisture conditions (Fig. 3, burrowed). Weight loss was monitored as a function of larval development from 14 mm to 32 mm body size.

RESULTS

Lethal temperature/survival capacity determinations

The ULT_{50} and LLT_{50} values for *T. obscurus* larvae are shown in Table 1. Dry conditions (12%) decrease resistance of this species to both high and low

Table 1. Upper (ULT_{50}) and lower (LLT_{50}) lethal temperature determinations for *Tenebrio molitor* and *Tenebrio obscurus* larvae under dry (12% RH) and moist (72% RH) conditions ($N = 40$)

Relative humidity (%)	Temperature (°C) *Tenebrio molitor** ULT	LLT	*Tenebrio obscurus* ULT	LLT	P† ULT	LLT
12	38.3	−4.5	39.2	−4.4	0.05	NS
72	43.8	−4.8	42.6	−5.9	0.05	0.05

NS = not significant.
*Data from Punzo (1975).
†Level of significance computed using Student's *t*-test.

temperature extremes. Under relatively moist conditions (72%), both ULT_{50} and LLT_{50} indicate an increased capacity for survival. Results indicate that RH has a greater effect on survival capacity at high temperature extremes than at low temperature extremes. Table 1 also compares upper and lower lethal temperature determinations for *T. molitor* and *T. obscurus* larvae. At cold temperature extremes, RH humidity has a minimal effect on *T. molitor* larvae. However, the opposite effect is found for *T. obscurus* larvae; relatively moist conditions (72%) increase the survival capacity at cold temperature extremes. At 72% RH, the ULT_{50} is higher for *T. molitor*, indicating that this species may survive to a greater extent than *T. obscurus* under these conditions. However, *T. obscurus* exhibits a higher LLT_{50} at 72% RH, suggesting that this species is better adapted to cold temperature regimes under relatively moist conditions.

The effects of temperature and RH on the survival capacity of *T. obscurus* larvae are shown in Figs 1 and 2. The results in Fig. 1 indicate that at cold temperatures, relatively moist conditions enhance survival. Under dry conditions, the larvae do not exhibit the same tolerance for temperature as that exhibited at 72% RH. Therefore, 12% RH represents a stressful environmental condition at cold temperature extremes.

The results in Fig. 2 show the effects of temperature and RH on larvae at high temperature extremes. Once again, 12% RH proves to be a stressful condition for survival. This figure differs from Fig. 1 by showing a more dramatic change in survival capacity at high temperature under relatively moist conditions (72%). There is a marked increase in the present survival between 42 and 43°C. Results show that the combination of high temperature and 72% RH has a greater effect on the survival capacity of this species than does the combination of cold temperature and 72% RH. A Wilcoxon Sign Rank test (Winkler and Hays, 1975) showed the effects of RH (12 vs 72%) on survival capacity to be significant over the low temperature range ($Z = 2.12$, $P < 0.01$) and high temperature range ($Z = 2.52$, $P < 0.01$).

Table 2. Mean weight loss (mg/cm²) for *Tenebrio obscurus* larvae desiccated at 4 relative humidities over a period of 48 hr ($N = 15$)

Period of exposure (hr)	Relative humidity (%) 2	33	72	98
12	0.73	0.79	0.91	1.01
24	0.89	0.90	0.94	1.06
36	1.32	1.24	1.11	1.26
48	1.57	1.34	1.37	1.39

Table 3. Mean weight loss (mg/cm²/hr × 10) for *Tenebrio obscurus* larvae as a function of relative humidity and period of exposure ($N = 15$)

Period of exposure (hr)	Relative humidity (%) 2	33	72	98
12	0.67	0.77	0.89	0.92
24	0.71	0.70	0.73	0.72
36	0.68	0.69	0.65	0.67
48	0.69	0.71	0.71	0.64

Evaporative water loss/cuticular permeability

The results listed in Table 2 show the effects of moisture on weight loss. Under dry air conditions, these larvae show cuticular permeabilities that fall within the same range as those reported for xeric-adapted arachnids (Warburg *et al.*, 1980), insects (Cohen, 1982; Cohen *et al.*, 1981) and the more mesic spiders, *Argiope aurantia* and *Lycosa avida* (Punzo and Jellies, 1983). Under higher humidity conditions (72 and 98%), these larvae exhibit cuticular permeabilities that compare favorably with those reported for some mesic arthropods (Edney, 1977; Hadley *et al.*, 1981). Table 2 also indicates that weight loss within the first 12 hr was greater at higher humidities. These results suggest that cuticular permeability becomes more linear as saturation deficit increases. The data listed in Table 3 indicate that rates of water loss decrease following initial loss. This compares favorably with data reported for other terrestrial arthropods (Edney, 1977) and indicates that the rate of

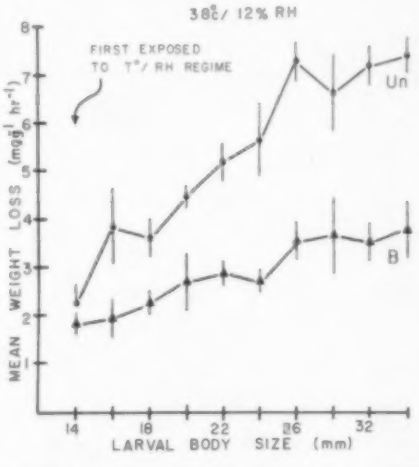

Fig. 3. Comparative weight loss rates of burrowed (B) and unburrowed (Un) *Tenebrio obscurus* larvae during larval development. Each datum point represents the mean plus or minus standard error. Larvae were weighed individually at exposure to 38°C and 12% RH.

evaporate water loss is clearly associated with the integrity of the cuticle.

The results of Fig. 3 show the effects of fossoriality on water loss as a function of development for *T. obscurus* larvae. Water loss for those larvae allowed to burrow (Fig. 3, burrowed) was found to be higher than that exhibited by unburrowed larvae (Fig. 3, unburrowed). Analysis of variance showed the differences in water-loss between burrowing and non-burrowing larvae to be significant ($F = 9.03$, $P < 0.01$).

DISCUSSION

The survival of terrestrial animals, to a large extent, is intimately associated with the capacity to resist desiccation through evaporative water loss. Terrestrial arthropods lose water through the cuticle and/or respiratory surfaces. Since temperature, as well as RH have an effect on water loss, water–temperature relationships represent a vital parameter with respect to the survival capacity. The integument is the main avenue for water loss, and the degree of permeability of the general cuticle determines the rate of water evaporation. Another factor affecting water efflux is the pressure gradient in the air surrounding the arthropod. If the air is nearly saturated with water, it is nearly impossible for water to pass from the cuticle into the atmosphere. Under very dry conditions the gradient is such that water is more easily transpired across the cuticle. The physiological relevance of cuticular permeability is clear. It is possible to think of a particular permeability characteristic of the cuticle as being the result of adaptation. Activity and distribution of terrestrial arthropods is influenced by the ability of the organism to compensate for evaporative water loss. The degree of resistance to evaporative water loss is a function of where an organism lives. Cohen (1982b) reported on two hemipteran species that live in arid habitats. Results indicated that the thermal tolerance of *Geocoris punctipes* was considerably higher than that of *Lygus hesperus*; however, *L. hesperus* exhibited a higher resistance to water loss at high temperatures. These results were related to the ecology of these two insects: *G. punctipes* lives on or in the soil where RH is higher, whereas *L. hesperus* is found above the ground where RH is markedly lower. The results reported by Punzo and Jellies (1983) on araneid and lycosid spiderlings showed that the cuticular permeability of arthropods from mesic habitats can be several orders of magnitude higher than that of xeric species. The results suggested that the rate of evaporative water loss is a property of the cuticle as well as the drying power of the air.

Species of *Tenebrio* are generally found in decaying vegetation where moisture conditions are relatively high. In its ecology, *T. obscurus* (as well as *T. molitor*) differs considerably from all but a few members of this genus in that it frequents stored grain bulks. Although there are numerous species of xeric-adapted tenebrionids, one does not normally find *T. obscurus* or *T. molitor* in desert regions. Generally, they are mesic species that, at some time during their life cycle, may invade granaries which can reflect xeric habitats to a large extent (Punzo and Mutchmor, 1980).

In this study, *T. obscurus* larvae exhibited a higher ULT_{50} and LLT_{50} under moist conditions. This would suggest that a granary characterized by dry conditions might not represent an ideal habitat for this species. However, larval survival at high and low temperature extremes under dry conditions (12% RH) was rather high although reduced from that at 72% RH (Figs 1 and 2). Although little comparable information is available for other species of *Tenebrio*, studies reported by Punzo and Mutchmor (1978, 1980) on the effects of temperature, moisture and period of exposure on the survival capacity of *T. molitor* allow for some comparisons to be made. These studies showed that high and low temperature extremes were stressful under dry (12% RH) conditions as well as moist (75, 98%) conditions for younger (7–10 mm) larvae. Older (larger) larvae, however, responded differently. High and low temperature extremes under moist conditions were not stressful, whereas under dry conditions, the higher temperatures (30°C) were somewhat stressful. The younger larvae cannot withstand high and low temperature extremes at 12% RH because they desiccate. They cannot use evaporative cooling because they cannot afford to lose an already limited supply of tissue water due to small body size. The older (larger) larvae of *T. molitor* can tolerate some water loss and thus exhibit a higher survival capacity at temperature extremes under dry conditions. Similar results were found for *T. obscurus* larvae in the present study. It was also found that, at 72% RH, younger *T. molitor* larvae exhibited lower tolerance at higher temperatures (Punzo and Mutchmor, 1978). With the older larvae, high temperatures caused a higher mortality rate than did low temperatures. However, under high RH conditions (98, 72%), evaporative water loss was reduced and the limiting effects of high temperature cannot be as successfully counteracted.

The present study analyzed the effects of temperature and RH on the survival capacity of older (14–32 mm) larvae of *T. obscurus*, the stage which is most destructive to stored grain. The results of this study suggest that RH *per se* has a marked effect of larval lethal temperatures (Table 1), survival capacity (Figs 1 and 2) and cuticular permeability (Tables 2 and 3). Similar moisture effects on survival capacity have been reported for the closely-related tenebrionid, *T. molitor* (Punzo and Mutchmor, 1980), as well as for other Coleoptera (Blumberg, 1971; Cloudsley-Thompson, 1970; Cohen *et al.*, 1981; Riddle, 1981) and additional terrestrial arthropods (Hadley *et al.*, 1981; Jungreis *et al.*, 1982; Punzo and Jellies, 1983; Warburg *et al.*, 1980). Although the water content of arthropod cuticles can vary between species, it is relatively low. Thus, arthropods whose cuticle constitutes a larger proportion of their total body weight (such as Coleoptera) will exhibit lower water contents and be more susceptible to desiccation via evaporative water loss.

The effects of RH on cuticular permeability (Tables 2 and 3) suggest that water loss is initially greater under high RH conditions for the larvae of this species. Similar results have been reported for other terrestrial insects (Cohen, 1982a; Cohen *et al.*, 1981;

Wilson *et al.*, 1982) as well as for the troglobitic spider, *Lycosa howarthi* (Hadley *et al.*, 1981). However, rates of water loss were found to be independent of RH in an undescribed epigean spider (Hadley *et al.*, 1981).

One of the most important behavioral thermoregulatory mechanisms exhibited by arthropods is fossoriality. This mechanism is especially pronounced in xeric-adapted species. Fossorial habits of xeric arthropods are well documented (see reviews by Cloudsley-Thompson, 1970; Edney, 1977). Burrowing is also an important factor in the ecology of granivorous insects that infest cereal products (Punzo and Mutchmor, 1978, 1980). Fossoriality can also result in a decrease in evaporative water loss. The differences in water loss exhibited by burrowing vs non-burrowing *T. obscurus* larvae (Fig. 3) under conditions of high temperature stress clearly attest to the effectiveness of this strategy. Jungreis *et al.* (1982) reported similar results for larvae of the tobacco hornworm, *Manduca sexta*. In this lepidopteran species, differences in rates of water loss between burrowed and unburrowed larvae were clearly evident for 2–4 days after larval (5th instar) wandering but were less pronounced shortly before pupation (day 5). In the present study, these differences became more pronounced as larval development progressed, especially between the 22–32 mm stages.

Although *T. obscurus* is not a true desert species, the results of this study indicate that it can adapt to a xeric-like environment. Desert species have evolved in arid environments and are frequently characterized by a well-developed and highly chitinized cuticle (Hadley, 1982, 1980) as well as other means of retaining water such as the apical membrane of the epidermal cells underlying the cuticle (Punzo and Jellies, 1983). In a study of two sympatric species of desert cockroaches (Cohen, 1982a), it was found that *Arenivaga investigata* inhabited a hotter and drier micro-habitat than *A. apacha*. However, the rates of water loss were higher in *A. apacha*. This was explained by the fact that *A. apacha* lives on or near the soil where temperatures and RH are more moderate, and its rate of water loss is comparable to mesic cockroaches. At some stage during its life cycle, *T. obscurus* is able to invade a xeric-like environment such as stored grain. Although no data concerning the temperature/water relations are available for all life cycle stages of this species, it would appear that older larvae are better adapted to granaries based on studies of other stored-product insects (Amos and Waterhouse, 1969; Bailey, 1969; Fahy, 1971; Punzo and Mutchmor, 1980). Older larvae are larger and possess a thicker, more sclerotized cuticle. Younger larvae have more translucent cuticles with a reduced degree of sclerotization. This may be one reason why they cannot as effectively block the passage of water under dry conditions as can the older larvae (Punzo, 1975; Punzo and Mutchmor, 1980). Under high stressful temperatures and 12% RH, upper and lower lethal temperature determinations (Table 1) suggest that *T. obscurus* ($ULT_{50} = 39.2$°C) is better adapted for xeric conditions than *T. molitor* ($ULT_{50} = 38.3$°C).

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THE *IN VITRO* EFFECTS OF PHOSPHODIESTERASE INHIBITORS ON 17α,20β-DIHYDROXY-4-PREGNEN-3-ONE INDUCED GERMINAL VESICLE BREAKDOWN IN BROOK TROUT (*SALVELINUS FONTINALIS*) OOCYTES

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Abstract—1. The *in vitro* effects of the phosphodiesterase inhibitors, SQ 20,006 and 3-isobutyl-1-methylxanthine (IM-xanthine), on 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-PG)-induced germinal vesicle breakdown (GVBD) were investigated in brook trout (*Salvelinus fontinalis*) oocytes.

2. Both SQ 20,006 and IM-xanthine inhibited 17α,20β-PG-induced GVBD *in vitro* though IM-xanthine was clearly more effective.

3. IM-xanthine was effect in inhibiting GVBD when added from 0 to 20 hr following 17α,20β-PG stimulation.

INTRODUCTION

Several reports have demonstrated that phosphodiesterase inhibitors or cAMP can inhibit progesterone-induced meiotic maturation of amphibian oocytes (Morrill *et al.*, 1972; Ozon *et al.*, 1978). Further, a decrease in cAMP is observed following progesterone stimulation (Morrill *et al.*, 1981). Thus, it appears that progesterone may stimulate meiotic maturation in amphibian oocytes by lowering cAMP and the decrease is most likely a result of the inhibition of adenylate cyclase (Finidori-Lepicard *et al.*, 1981).

Steroids also induce meiotic maturation in fish oocytes; however, the naturally-occurring maturational steroid is probably different than in amphibians (Goetz, 1983; Masui and Clark, 1979). There are similarities in the steroidal induction of meiotic maturation in fish and amphibians which suggest that the mechanism of action of the steroid would be similar in both vertebrates. For example, steroid-induced meiotic maturation in oocytes of both vertebrates can be inhibited by translational but not transcriptional inhibitors (Goetz, 1983; Masui and Clark, 1979). To our knowledge, no reports have been published concerning the effects of phosphodiesterase inhibitors or cyclic nucleotides on germinal vesicle breakdown (GVBD) in fish oocytes. In view of the interesting mode of action that progesterone may have in the stimulation of meiotic maturation in amphibian oocytes, it would be important to determine if a similar mechanism occurs in fish.

In this report we demonstrate that phosphodiesterase inhibitors block 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-PG)-induced GVBD in intrafollicular brook trout (*Salvelinus fontinalis*) oocytes.

MATERIALS AND METHODS

Ovaries were obtained from gravid brook trout (100–200 g) during the reproductive season (September–October). *In vitro* incubations of intrafollicular oocytes were conducted as previously described (Duffey and Goetz, 1980; Goetz and Bergman, 1978) except that HEPES (*N*-2-hydroxyethyl-piperazine-*N*′-2-ethane-sulphonic acid) (0.02M) was added to the incubation medium (Cortland-Wolf and Quimby, 1969) and the pH adjusted to 7.4 with 1 N NaOH. Incubates, containing 15 oocytes/flask, were maintained at 12°C for 48 hr prior to assay and were intermittently shaken. The phosphodiesterase inhibitors, 3-isobutyl-1-methyl-xanthine (IM-xanthine-Sigma) and SQ 20,006 (1-ethyl-4-hydrazino-1H-pyrazolo [3,4b] pyridine-5-carboxylic acid, ethyl ester, hydrochloride-Squibb) were dissolved directly in the incubation medium. Oocytes were assayed for GVBD in a fixative previously described (Goetz and Bergman, 1978).

RESULTS

Figures 1 and 2 show the effects of two phosphodiesterase inhibitors, IM-xanthine and SQ 20,006, on 17α,20β-PG-induced GVBD in brook trout oocytes. Stimulatory concentrations (2.5 and 5.0 ng/ml) of 17α,20β-PG were chosen on the basis of past dose–response curves for brook trout (Duffey and Goetz, 1980) and represent levels that would theoretically be high enough to just stimulate 100% GVBD depending on the sensitivity of the oocytes. In all experiments, these 17α,20β-PG levels did stimulate 100% GVBD when incubated without inhibitors (Figs 1 and 2 and Table 1). Both IM-xanthine and SQ 20,006 inhibited 17α,20β-PG-induced GVBD; however, IM-xanthine was more effective. Whereas IM-xanthine completely blocked GVBD at 1.0 and 0.1 mM, SQ 20,006 was completely inhibitory only at 1.0 mM. Controls with inhibitor alone or without inhibitor or steroid never matured.

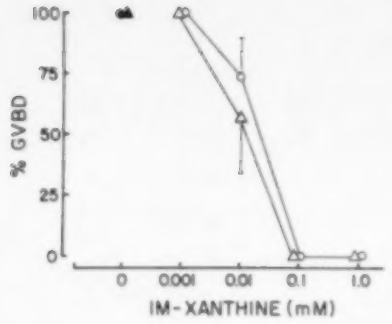

Fig. 1. *In vitro* effects of 3-isobutyl-1-methyl xanthine on 17α,20β-PG-induced GVBD ($\bar{X}$% ± SEM, $N = 3$). Open circles and triangles indicate IM-xanthine effect with 5.0 and 2.5 ng/ml 17α,20β-PG respectively. Closed circle and triangle indicate % GVBD with 5.0 and 2.5 ng/ml 17α,20β-PG alone.

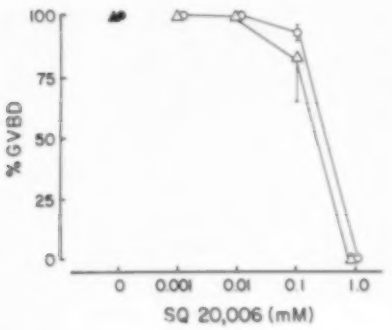

Fig. 2. *In vitro* effects of SQ 20,006 on 17α,20β-PG-induced GVBD ($\bar{X}$% ± SEM, $N = 3$). Open circles and triangles indicate SQ 20,006 effect with 5.0 and 2.5 ng/ml 17α,20β-PG respectively. Closed circle and triangle indicate % GVBD with 5.0 and 2.5 ng/ml 17α,20β-PG alone.

When IM-xanthine was added to the incubation medium every four hours (for 24 hr) following 17α,20β-PG stimulation (5.0 ng/ml), complete inhibition was observed with 1.0 mM until 24 hr (Table 1). At lower IM-xanthine concentrations (0.1 mM) inhibition was complete until 20 hr.

Table 1. *In vitro* effects of 3-isobutyl-1-methyl-xanthine (IM-xanthine) added to brook trout incubates at various times following 17α,20β-PG (5.0 ng/ml) stimulation

Time of IM-xanthine Addition (hr)	IM-xanthine (mM) 1.0	0.1
0	0*	0
4	0	0
8	0	0
12	0	0
16	0	0
20	0	27.2
24	42.8	72.2

All incubates assayed at 48 hr. In incubates with 17α,20β-PG alone, 100% GVBD was recorded.
*$\bar{X}$% GVBD of two replicates on oocytes from one donor.

DISCUSSION

The results of this investigation clearly demonstrate that phosphodiesterase inhibitors can block 17α,20β-PG-induced GVBD in intrafollicular brook trout oocytes. These results are similar to those reported for amphibians, in which phosphodiesterase inhibitors blocked progesterone-stimulated GVBD (Morrill *et al.*, 1972; Ozon *et al.*, 1978). However, as far as we know, they are the first results of this type reported for fish oocytes. Further studies will be necessary to determine if the inhibition is a result of cyclic nucleotide accumulation, since other mechanisms for the inhibition of meiotic maturation by phosphodiesterase inhibitors have been suggested (O'Connor and Smith, 1976).

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